Anthropometric measurements, sexual development and serum reproductive hormonal levels among boys in the rural Western Cape

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DECLARATION

I, Jun Mao, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Acknowledgments

My deepest gratitude goes first and foremost to my supervisor Professor Mohamed Aqiel Dalvie from University of Cape Town's Center for Occupational and Environmental Health Research, for his constant encouragement and guidance throughout 2014 and 2015. He has walked me through all the stages of the development of this master dissertation. Without his consistent and illuminating instruction, this dissertation could not have reached its present form. During my master's study, Professor M.A Dalvie has been an important mentor who contributed a great deal of support on my professional skill and career development. Also, I am particularly grateful for his financial assistance to help me complete my Master's study.

Second, my thanks would go to my beloved family for their support and great confidence in me all through these years. Last, I also owe my sincere gratitude to my friends and my fellow classmates who gave me their help and time in listening to me and helping me work out my problems during the difficult course of the dissertation.

PART A: RESEARCH PROTOCOL

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1 INTRODUCTION

1.1 BACKGROUND

1.1.1 PUBERTAL DEVELOPMENT

Puberty is a complex process, rather than a single event. It is characterized by adolescent growth spurt, rapid changes in body composition, development of the secondary sex characters, maturation of the gonads, achievement of fertility and behavioral and psychological changes. These series of events constitute a critical period when a child's body matures into an adult body and achieving fertility. Pubertal development can be measured in different ways including anthropometric evaluation, secondary sexual characteristics assessment, and hormonal measurement (Hayward, 2003). These different measurements provide valuable assessment tools for studying and monitoring pubertal development of the population.

1.1.2 SEXUAL MATURATIONAL RATING

Sexual maturational rating is the most important tool to measure the process of puberty. Unlike other growth and development periods, the series of pubertal events correlate highly with the adolescents' degree of biological maturation which cannot be simply indicated by age. Adolescents with earlier biological maturational levels will be at a

more advanced pubertal stage than those who mature late, (Rogol *et al.*, 2000). Furthermore, in health practice sexual maturational assessment has been commonly used as an indicator for biological maturation. Pubertal sexual maturation occurs under the influence of both adrenarche and gonadarche. Adrenarche is the process in which there is increased ncreased release of adrenal androgen in males, which results in pubic and axillary hair development in boys. Gonadarche is characterized by the development of the gonads associated with increased production of the gonadal steroid hormones, primarily testosterone in boys, which cause testicular enlargement in boys. Biological maturation has been classified into five stages by Marshall and Tanner (Marshall & Tanner, 1970), known as "Tanner Stages", Table 1.

Table 1 Tanner Stages for Males

Stage	Genital Development	Pubic Hair Growth
1	Prepubertal; no change in size or proportion of testes, scrotum and penis from early childhood	Prepubertal; no pubic hair
2	Enlargement of scrotum and testes; reddening and change in texture in skin of scrotum; little or no penis enlargement	Sparse growth of hair at base of penis
3	Increase first in length then width of penis; growth of testes and scrotum	Darkening, coarsening and curling, increase in amount
4	Enlargement of penis with growth in breadth and development of glands; further growth of testes and scrotum, darkening of scrotal skin	Hair resembles adult type, but not spread to medial thighs
5	Adult size and shape genitalia	Adult type and quantity, spread to medial thighs

Source: Data from Tanner JM. Growth at adolescence. Oxford: Blackwell Scientific Publications, 1962

1.1.3 PUBERTAL TIMING

The initiation of puberty remains poorly understood, but there is consensus that the first sign of secondary sexual development can be regarded as the onset of puberty. Knowledge of the timing of puberty is of continuing social and public health interest (Jones *et al.*, 2009) as it not only relevant for social, behavioral and educational development of the individual, but also for long-term health risks, including obesity, reproductive and mental health (Johansson & Ritzén, 2005; Mendle *et al.*, 2007).

Numerous studies on age at the onset of puberty have been conducted worldwide and have it is influenced by factors such as race, ethnicity, nutritional conditions and

geographic location can influence the age at the onset of puberty (Van Wieringen, 1978; Eveleth 1978; Herman-Giddens *et al.*, 2001; Natale & Rajagopalan, 2014).

An association between nutritional status and onset of puberty was initially made when it was noticed that there was a secular decline in the age at onset of puberty which was linked to rapid economic development in many developed countries (Olesen *et al*., 2000; Hwang *et al.*, 2003; Łaska-Mierzejewska & Olszewska, 2004; Onland-Moret *et al.*, 2005). The impact of nutritional status on onset of puberty was further demonstrated in numerous epidemiological studies on children who were adopted from developing countries and raised in Western Europe and United States (Proos *et al.*, 1991; Bourguignon *et al.*, 1992; Virdis *et al.*, 1998; Krstevska-Konstantinova *et al.*, 2001; Teilmann *et al.*, 2002). The children adopted in developing countries was raised in a higher socio-economic setting than those raised in underprivileged settings in developing countries from Asia, Africa and South-America and their onset of puberty occurred much earlier (Kulin *et al.*, 1982; Natale & Rajagopalan, 2014; Chompootaweep *et al.*, 1997; Macías‐Tomei *et al.*, 2000; Carrillo *et al.*, 2001; Ma *et al.*, 2011).

Recent studies, however, have found an unexpected new decline in the age at onset of puberty mostly among females in the past 15 years in developed countries, which was unlikely to have resulted from any major changes in socio-economic conditions (Sun *et al.*, 2002; Wu *et al.*, 2002; De Simone *et al.*, 2004; Castellino *et al.*, 2005; Aksglaede

et al., 2009). Here, it is thought that exposures to chemicals with endocrine-disrupting properties via food and environment may play a role. Many laboratory experiments suggested that endocrine disrupting chemicals (EDCs) exhibits hormonal or anti-hormonal activity, usually oestrogenic or anti-androgenic (Damstra *et al.*, 2002; Sharpe & Irvine, 2004). EDCs can affect the HPG axis on receptor level or influence the synthesis and metabolism of hormones, receptors and transcription factors which can impact on sexual development and functioning (Takeyoshi *et al.*, 2002; Toppari, 2002; Rasier *et al.*, 2006). However, the effects of EDC's on pubertal development are difficult to investigate in epidemiological studies as it is difficult to separate it from the effects due to differences in nutritional status. Additionally, toxicological studies have shown that the effects of EDCs are highly dependent on the period of exposure, exposure dosage, duration of exposure, and half-life of the compounds.

1.1.4 PUBERTAL GROWTH SPURT

The pubertal growth spurt which is one of the main features of puberty, is characterized by significant somatic growth and measurable changes in body size and body composition. From the onset of puberty, the rate of growth in height increases intensively until it reached the peak height velocity in mid-puberty, and then followed by a sharp decline and completion by the end of puberty. Besides growth in height, about 50% of the adult body weight is gained during puberty (Barnes, 1975; Marshall, 1978).

1.1.5 PUBERTAL GROWTH PATTERN IN UNDERNOURISHED CHILDREN

Periods of accelerated growth may create a special need for nutrition, and the growth of children reflect their nutritional status. The anthropometric parameters of children with low nutritional status during pre- and early puberty are generally characterized by shorter statue and lower weight in comparison to those with high nutritional status (Graham *et al.*, 1980; Papadimitriou *et al.*, 2002; Eiben *et al.*, 2005; Schwekendiek, 2009; Omigbodun *et al.*, 2009). Despite the fact that early pubertal growth might be sensitive to the insults imposed by malnutrition, puberty may also serves as catch-up periods for regaining the previous growth loss. For example, in a cross-sectional survey of for adolescents conducted in Mpumalanga, the prevalence of stunting in height as per 2006 WHO growth reference,was highest (9 %) at Tanner Stage 1 for both girls and boys and then reduced to about 1% at Tanner stage 5 (Kimani-Murage *et al.*, 2010). Similarly, in a cross-sectional study conducted in South-Western Nigeria, the prevalence of stunting in rural boys fell from 33.4% at age of 14 years to 18.6% at age 19 years (Omigbodun *et al.*, 2010).

Pubertal catch-up growth may occur because of food supplementation which in turn could be the result of preferential feeding, public nutritional programs, and/or adolescents sourcing food themselves. The magnitude of catch-up may depend on factors such as food shortage that that deteriorate growth (Graham *et al.*, 1980). A study of the pubertal growth pattern in a particular population can provide valuable data for

pubertal health promoting programs such as nutritional interventions in the community.

1.1.6 MALE REPRODUCTIVE HORMONAL SYSTEM

HPG axis which is the major regulator of the mature reproductive system, remain quiescent during childhood and is reactivated at the onset of puberty (Lee *et al.*, 1974). The reactivation of HPG cause fluctuations in hormones levels resulting in variable downstream effects on the body. The hypothalamus produces gonadotropin-releasing hormone (GnRH) which stimulates the pituitary gland to secrete gonadotropins such as luteinizing hormone (LH) and follicle–stimulating hormone (FSH), which regulate the production of sex steroids, predominately testosterone and estrogen, by the gonads. LH stimulates the production of testosterone by Leydig cell, while FSH stimulates inhibin production in the testis, activates the Sertoli cells and induces the maturation of spermatocytes. Testosterone simulates the growth of male reproductive tissues and promotes secondary sexual characteristics, and estrogenic steroid hormones induce the maturation of the sperm and maintain a healthy libido. In males, oestrogen is converted from testosterone by aromatization.

Since the onset of puberty, the progression of puberty is associated with rapid fluctuations in gonadotropins and sex steroids levels. These alternation in hormones play a vital role in supporting the development of reproductive organs, secondary sexual characteristics and maintaining normal reproductive function. Clinical disorders of the gonadotropins and sex steroids synthesis and action will not only results in abnormal

of growth but also have irreversible negative impact on reproductive health (Perry *et al.*, 2008). However, only few studies in the literature that have investigated the alternation of the reproductive hormones during the periods of pubertal development, and most of these studies were conducted in countries from Europe (Andersson *et al.*, 1997; Raivio, *et al.*, 1998; Crofton *et al.*, 2002; Chada *et al.*, 2003).

1.1.7 REPRODUCTIVE HORMONES AS MARKERS OF REPRODUCTIVE HEALTH IN MALES

Reproductive hormones are critical to spermatogenesis and maintenance of reproductive function in men (Meeker *et al.*, 2008). In adults, serum FSH and inhibin B levels have long been recognized as indicators of spermatogenesis. Many studies have found that sperm concentration is positively associate with inhibin B levels, and inversely associated with FSH levels (Jensen *et al.*, 1997). In a study of 349 Danish men who had not previously attempted to make their wifes pregnant, have reported that the predictive power of detecting oligospermia (sperm concentration below 20 mill/ml) among men who have inhibin B below 80 pg/ml and FSH above 10 IU/L, was 100% (the predict power for each hormone alone was 80.0% and 85.7% for inhibin B and FSH, respectively) (Jensen *et al.*, 1997).

The predictability of inhibin B and FSH on semen quality was suspected to be due to their ability to reflect the quantity of Sertoli cells which determine both testicular size and sperm production (Orth *et al.*, 1988; Anderson & Sharpe, 2000). Puberty is critical

period for Sertoli cell maturation as it determine the final quantity of Sertoli cells in adulthood. A previous study investigating the reproductive hormonal alternations during puberty have found that the fluctuation in the concentrations of serum inhibin B, FSH and testosterone levels coincide with critical stages of Sertoli cell maturation (Plant & Marshall, 2001) which indicates that the reproductive hormonal profile at puberty may predict the future reproductive health, particularly semen quality in adulthood.

1.2 PROBLEM STATEMENT AND JUSTIFICATION

Research data on biological maturity in local communities is important for providing insight and for planning of prevention initiatives of adult health problems (De Onis & Habicht, 1996). There are currently no reference data on anthropometric characteristics, reproductive hormones and sexual development in pubertal children from the rural Western Cape of South Africa. Considering the fact that these children are living under poor nutritional conditions and exposed to environmental chemicals including endocrine disrupting chemicals, there is an urgent need for such data.

1.3 RESEARCH QUESTIONS

1. What is the relationship between serum reproductive hormonal levels (LH, FSH, testosterone, estradiol) and sexual development (Tanner stage score) among boys in the rural Western Cape in South Africa?

2. What are the reproductive hormonal levels, anthropometric characteristics and sexual maturity ratings of boys in the rural Western Cape in SA?

3. What is the relationship between anthropometric measurements (height, weight, BMI) and sexual development (Tanner stage score) among boys in the rural Western Cape in South Africa?

4. What is the relationship between serum reproductive hormonal levels (LH, FSH, testosterone, estradiol) and sexual development (Tanner stage score) by Tanner stage among boys in the rural Western Cape in South Africa?

1.4 RESEARCH OBJECTIVES

a) To characterize the demographic and reproductive health information of the participants.

b) To measure the weight, height and body mass index of the participants.

c) To evaluate the physical sexual maturity of the participants.

d) To measure the baseline reproductive hormones levels of the participants.

e) To measure confounders to the various reproductive health outcome such as low birth weight, mother's habits during pregnancy, lifetime phytoestrogen intake, and dietary content of soya beans and other vegetables.

f) To investigate the effect of the sexual maturity progression on the reproductive hormones levels of the boys while controlling for relevant confounders.

g) To investigate the relationship between the sexual maturity progression and anthropometric variables while controlling for relevant confounders.

2 METHODS

2.1 STUDY DESIGN

This thesis is a sub-analysis of a dataset from a study which was done between April 2007 and March 2008. The study was a cross-sectional analytic study that investigated the effect of environmental pesticides exposure on growth, pubertal sexual development and endocrine status of boys and adolescents in the rural Western Cape, South Africa (Appendix B).

2.2 STUDY POPULATION

The study population includes all boys who attended primary and secondary schools in three agriculturally intense and neighbouring non-agricultural rural areas in the Western Cape Province. These included the Hex River Valley, where grape farming is practised; Grabouw, where pome fruit farming is predominantly practised and; Piketberg, where wheat and fruit farming is practiced.

2.3 SAMPLING

The Western Cape Department of Education provided a list of schools in the selected areas. Boys from the most accessible primary and secondary schools that had learners from both farms and neighbouring towns, were recruited. Prior to the start of the study, the study co-ordinator and principle investigator engaged with the school principals and held meetings with the staff to inform them about the proposed study. Parents were asked in advance, by means of letters distributed to schools, to provide provisional written consent for their children to be recruited. Once the school and parents agreed

and the boys were recruited into the study, parents were informed of the details about when the study was to start and what was to be expected of them. Boys whose parents consented (n = 492) were stratified according to age, and according to whether they had lived on a farm or not at the time of the study. If the number of consenting boys living on farms exceeded the number of boys to be selected at a school, random systematic sampling was used to select the boys. The number of consenting non-farm boys did not exceed the target and therefore all of them were selected in the study. Selected boys and their parents or guardians were invited to participate in the study and were asked to present at the school on specified dates.

2.4 SAMPLE SIZE CALCULATION

The sample size was calculated based on the mean value (3.57 ± 3.15 miu/ml) of blood follicle stimulating hormone (FSH) levels which was found to be the most variable hormone in a previous published study (Dalvie, 2002) investigating the reproductive health effects under the dichlorodiphenyltrichloroethane (DDT) exposure. A two-sample test of equality of means by Stata 12 statistical software (StataCorp, Texas, USA) indicated a sample size of 174, split as 116 from farm and 58 from non-farm assuming an 80% power, and a 40% difference in blood follicle stimulation hormone (FSH) and a confidence level of 95%.

Inclusion criteria

Only boys aged 5 -19 years living in rural Western Cape in South Africa in the 3 study

areas were included in the study.

Exclusion criteria

There were no exclusion criteria. All boys selected within the age categories who agreed to participate were included in the study

2.5 INSTRUMENTS

2.5.1 QUESTIONAIRE

Trained interviewers conducted face-to-face interviews with the parent or legal guardian and boy in their language of preference. The responses were recorded directly onto an electronic questionnaire which was pre-loaded onto a cell phone using mobile technology (Mobile Researcher, Clyral). At the end of each interview the completed questionnaires were downloaded onto a central website and were accessed by the Principal Investigator. This information was then exported into Microsoft Excel® and Stata 10 [Stata Corporation, Texas, USA] for further data management and analysis.

The questionnaire was developed by the study team, led by the Principal Investigator, and was based on previous local studies in similar populations. English, Afrikaans and Xhosa versions of the questionnaire were developed. The latter questionnaires were back-translated to English during their development to ensure validity and reliability of the questions. A copy of the questionnaire can be viewed in Appendix B. The questionnaires were administered by fieldworkers, who were extensively trained in

conducting interviews, on the content, and on the various terminologies in the questionnaire. The questionnaire included questions on: demographics, lifetime residency on farm or town, birth weight, general medical history, genital health history and lifetime environmental exposure to pesticides (diet and dietary content of soya beans and other vegetables, mothers' personal habits during pregnancy (alcohol consumption, smoking, diet, and the use of soya milk after conception or birth)., domestic use of pesticides, domestic water sources, use of empty pesticide containers. The interviewers administered the questionnaires to the parent regardless of the child's age.

2.5.2 PHYSICAL EXAMINATION

A trained male nurse conducted a physical examination of the boy recording the information onto a structured record form (Appendix D). The nurse measured sexual maturity rating (SMR) according to the Tanner scoring system and also recording genital anatomical abnormalities (congenital hydrocoeles, undescended testes, congenital inguinal hernias and hypospadias, the presence of infection, previous injury or tumours, testicular consistency and size). SMR was derived by assessing penis development (testicular maturation) and then determining a score using Tanner stages, but with stages five and six merged. Testicular volume was also assessed using a standardized set of wooden testicular beads [Orchidometer, Kabi, Japan]. Height and weight were also recorded according to standardized methods, and using calibrated instruments. Photographs showing the various Tanner Stages were used as a reference (Appendix C). The nurse was trained (2 days) by a local reproductive paediatrician

demonstrating how to perform the anthropometric measurements, use the orchidometer and assess the sexual maturation score by using pictures and through demonstration on a boy patient.

2.5.3 BLOOD ENDOCRINE MEASUREMENTS

The nurse drew blood (2 X 6 ml venous blood) from the boys as early as logistically possible, preferably before 13h00. The blood samples were allowed to clot, and were then centrifuged at 5000 revolutions per minute in the field (using a portable centrifuge) by a qualified laboratory technician. The serum samples were stored in an in-field refrigerator and then transported on ice to the National Health Laboratory Sciences (NHLS) facility at Groote Schuur Hospital in Cape Town within 24 hours. The NHLS analysed serum samples for baseline FSH, luteinizing hormone (LH), testosterone, oestradiol (E2), and serum hormone binding globulin (SHBG). LH was measured with the MAIAclone IRMA kit (Bologna, Italy) [Biochem Immuno Systems, 1995], FSH and total testosterone with ACS-180 competitive chemiluminescent automated systems (New York, USA) [Bayer Corporation, 2000], oestradiol with an in-house radioimmunoassay (Nivellus, Belgium) [Biosource-Europe SA,1994], and SHBG with the IRMA kit from Orion Diagnostica (Finland) [Orion Diagnostica, 1990]. Baseline hormonal levels were compared to age-related laboratory normal ranges.

2.6 PILOT STUDY

A pilot study was conducted (on 3 boys from the study areas) to field-test the questionnaire and the logistics for the main study in April 2008.

2.7 LOGISTICS

Arrangements were made with the schools and parents for a date and suitable venue to conduct the testing. The construction of the questionnaire and training of interviewers were conducted during January 2008 to March 2008. The main study was performed during May 2008 to July 2008. Laboratory analysis of biological samples, entering of data took place during May 2008 to August 2010. Data analysis took place during 2014 November to 2015 February. Final write-up took place from February 2015 to December 2015.

2.8 FIELD PROCEDURES

The team that conducted fieldwork consisted of a field coordinator, interviewers (n =3), a male nurse (who drew the bloods), a bio-technician (who processed the biological samples before it was sent to the laboratory), and a male nurse who did a physical examination of the boys.

The mothers were encouraged to be present for the interviews as it was assumed that they would be able to provide more reliable information regarding the boys' developmental and personal patterns, and their own personal and occupational habits during their pregnancies. On arrival, the parent or legal guardian signed the informed consent form in the language of their choice. The interviewer administered the questionnaire to the parent or legal guardian. Thereafter, the boys were examined by a

male study nurse. After the physical examination, the boy was seen by the second study nurse who drew the bloods. A technician then processed the blood samples and prepared it for transportation on ice to the laboratory for storage. These processes and procedures are described in more detail in the sections below.

3 ANALYSIS PLAN

3.1 DATA MANAGEMENT AND QUALITY ASSURANCE

All the completed questionnaires and data sheets were checked for completeness, consistency and logical reasoning by a single field supervisor in the field. A checklist on the outside of each envelope contained each participant's data. Where indicated, missing or relevant data were obtained before the interviewee and participant left the venue. Field workers were available in the study region to follow up on subjects who had missing or inconsistent questionnaire data, particularly for anthropometric and hormonal characteristics.

3.2 DATA ANALYSIS

All analyses will be performed using Stata 11 statistical software (StataCorp, Texas, USA).

Exploratory data analysis will be conducted first. Data for each variable will be examined for missing data, for any obvious abnormalities, and for the type of coding used. Normality for continuous variables will be assessed using histograms and Shapiro-Wilk tests. Numeric outcome variables will be categorized as described below.

Bivariate analysis will be conducted to test for the association between the outcomes and confounders using t-test for independent samples if data are normally distributed, or a Wilcoxon sum rank test if the data are skewed. Chi-square testing will be conducted

for categorical variables. A Fisher's exact test will be used when frequencies are less than 5. For associations between continuous variables, linear regression analysis techniques will be used.

Multiple Linear Regression Analysis will be used during multivariate analysis as all the outcome variables are continuous. Confounders will be selected on an a priori basis and will be based on biological plausibility and/or on bivariate testing (where associations with a significance of $p<0.1$ are found).

Regression diagnostics will be applied to determine the goodness of fit of the model. Collinearity will be assessed by calculating the simple correlation coefficients and variable inflation factors. Outliers and influential points will be assessed using regression diagnostic techniques. Studentised residual values of >2 will determine the presence of outliers. Influential points will be assessed by examining Cook's distance (0.33), dfit ($>2\sqrt{k/n}$) and dfbeta's ($>2/\sqrt{n}$). Descriptive data were explored and expressed as medians and interquartile intervals. Differences in age, anthropometric variables, testicular volumes and hormone concentration between successive Tanner stages were explored using logistic regression modelling while controlling for confounding and testing for interactions between the variables. Regression diagnostics to assess the form of the linear predictor and to assess for outliers or influential observations will be done. Outliers will be identified if the standardised residuals are >2 or <-2. Influential observations will be identified by determining the effect that the

covariate pattern has on the estimated model (>2(p/n)).

Anthropometric comparisons will be performed by using the 25th and 50th percentile for age from the CDC growth charts (Centre for Disease Control and Prevention, 2009) and the WHO growth reference (World Health Organizations, 2015) and then the results will be summarized according to Tanner stage.

Correlations between serum hormone levels (FSH, LH, testosterone and estradiol) and testicular volume were tested by Pearson correlation coefficient.

4 ETHICS

This study was done according to the Declaration of Helsinki of the 25th world Medical Assembly (WHO 2000). The ethic proposal of the study has been approved by the University of Cape Town's Research Ethics Committee (HREC REF 943/2014). The study respected participants' autonomy, the right to the information, privacy rights and confidentiality of their information. A copy of the written informed consent is attached as Appendix E.

4.1 BENEFIT

The findings of this study would have a population benefit that it would contribute a potential indication of the health of the pubertal male reproductive development. Such indication can serve for future implementation of strategies and possible health policies

to ensure the reproductive health of children who likely to expose to hormonally active agricultural pesticides in the rural area of Western Cape.

4.2 HARM/RISKS

Given the nature of the study, the physical examinations could be intimidating to the boys. The examinations were done under private conditions to ensure acceptability and confidentiality, in order to minimize the harm to the participants. The feelings of participants were respected during the interviews, all participants were enable making choices of not to answer questions which they were not comfortable with, or withdraw from the study at any time.

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PART B: LITERATURE REVIEW

1 INTRODUCTION

1.1 OBJECTIVE

The aim of the literature review is to review the status of the international literature on pubertal development of boys focusing on the relationship between physical reproductive development, sexual hormonal levels and anthropometric development. It also aims to identify the status of reference data for pubertal development in the international literature. The review provides a brief background of pubertal developmental events, such as reproductive development and the growth spurt. The review also describes the impacts from environmental factors such as nutritional conditions and chemical exposures. The literature review intends to examine the need for data on pubertal development in developing countries and in South Africa.

1.2 SEARCH STRATEGY

The literatures in this review includes peer reviewed articles from online and print journals and books. The search was limited to articles published in English and was conducted through Google Scholar as the main search engine. PubMed, Scopus and Medline was also used for key word searches. Key word search terms used include (Puberty) OR (Anthropometric variables) OR (Sexual development) OR (Pubertal hormones) OR (Reproductive development) OR (Growth Spurt) OR (Semen quality) OR (Tanner stage) OR (Reproductive hormones) OR (Endocrine disrupting chemicals) OR (weight, height and BMI). The scope of the review was as such that it only foscussed on epidemiological studies.

2 LITERATURE

2.1 PUBERTAL DEVELOPMENT

Puberty is a complex process, rather than a single event. It is characterized by adolescent growth spurt, rapid changes in body composition, development of the secondary sex characters, maturation of the gonads, achievement of fertility and behavioral and psychological changes. These series of events constitute a critical period when a child's body matures into an adult body and achieving fertility. Pubertal development is measured in different ways including anthropometric evaluation, secondary sexual characteristics assessment, and hormonal measurement (Hayward, 2003). These measurements are valuable for studying and monitoring pubertal development of boys.

2.2 SEXUAL DEVELOPMENT

Sexual maturation occurs under the influence of both adrenarche and gonadarche. Adrenarche is the process of increased release of adrenal androgen in males, which results in pubic and axillary hair development in boys. Gonadarche is characterized by the development of the gonads associated with increased production of the gonadal steroid hormones, primarily testosterone in boys, which cause testicular enlargement in boys. Adrenarche begins much earlier to the onset of puberty and other hormonal changes (Perry, 2008). It is not until the early stage of puberty approaches that the pubic hair becomes visible and with the progression of puberty, pubic hair continues to grow longer, darker and spreading over the base of the penis and becoming adult hair in late puberty. During this process, the testicles enlarge and the scrotum reddens indicating the start of gonadarche. The testes will grow from prepubertal volume of 3 ml or 4ml to a 10-fold increase in size by late puberty (Marshall, 1975). The penis enlargement (first growth in length and then in breadth) begins after testicular enlargement started and doubles in size during puberty (Marshall, 1975). These series of sexual development generally take places in a sequential order, but individual variation does occur normally. Based on these predictable sequence, timing, and tempo of events, Marshall and Tanner (1970) classified the secondary sexual characteristics development into five stages of changes, known as “Tanner stages” (See Table 1 Tanner Stages for Male, page 5). The “Tanner stages” range from stage 1 (prepubertal) to stage 5 (post-pubertal) to describe the growth of pubic hair, testicular and genital developments in boys. The standards for pubic hair and genital growth are given separately, because

usually the two develop with different timing (Marshall & Tanner, 1970).

Table 2 Tanner Stages for Males

Stage	Genital Development	Pubic Hair Growth
1	Prepubertal; no change in size or proportion of testes, scrotum and penis from early childhood	Prepubertal; no pubic hair
2	Enlargement of scrotum and testes; reddening and change in texture in skin of scrotum; little or no penis enlargement	Sparse growth of hair at base of penis
3	Increase first in length then width of penis; growth of testes and scrotum	Darkening, coarsening and curling, increase in amount
4	Enlargement of penis with growth in breadth and development of glands; further growth of testes and scrotum, darkening of scrotal skin	Hair resembles adult type, but not spread to medial thighs
5	Adult size and shape genitalia	Adult type and quantity, spread to medial thighs

Source: Data from Tanner JM. Growth at adolescence. Oxford: Blackwell Scientific Publications, 1962

2.3 PUBERTAL TIMING

The time to puberty is defined as the age at sexual maturation and it is generally used to identify early and late puberty. The time to puberty is of continuing social and public health interest, (Jones *et al.*, 2009) as it not only of relevance to social, behavioral and educational development individuals, but is also associated to long-term health risks, including obesity, reproductive and mental health (Johansson & Ritzén, 2005; Mendle *et al.*, 2007).

In boys, testicular growth represents the first sign of pubertal development, and it was proposed by Tanner & Marshall (1976) that the attainment of a testicular volume of 4 mL to be regarded as the criterion for assessing age of onset of puberty in boys. The commonly used markers of the onset of male puberty in epidemiological studies and health surveys, however, is Tanner genital Stage 2 (G2) (Ma e*t al.*, 2011) as it includes the initial testicular enlargement (Tanner, 1962; Grumbach & Styne, 1998; Parent *et al.*, 2003).

The age of onset of puberty among children is a topic which have received much attention in the past century (Herman-Giddens *et al.*, 2001; Anderson *et al.*, 2003; Gluckman & Hanson, 2006A; Gluckman & Hanson, 2006B). Numerous studies worldwide have shown that factors such as race, ethnicity, nutritional conditions and geographic location as well as exposure to endocrine disrupting compounds can influence the age at the onset of puberty (Van Wieringen, 1978; Eveleth 1978; Natale

& Rajagopalan, 2014; Herman-Giddens *et al.*, 2001).

Significant racial differences in the timing of sexual maturity was first noted among US children, when it was found that non-Hispanic black girls had earlier maturation than non-Hispanic white girls but no differences among boys of different races was found in the National Health Examination Survey (NHES), a nationally representative sample in US (Harlan *et al.*, 1979; Sun *et al.*, 2002). However, this NHES study only included children aged from 12 to 17 years old, which excluded the period of early sexual maturation. A later study on sexual maturity of children aged from 8 to 19 years (the Third National Health and Nutrition Examination Survey (NHANE III) conducted from 1988 to 1994 in US reported significant differences in the median age at onset of puberty among non-Hispanic black (9.2 years old), non-Hispanic white (10.0 years old), and Mexican-American (10.3 years old) boys (Sun *et al.*, 2002). The latter study also found that the age when US children completed their sexual development were approximately the same among different races which indicated that there were less differences amongst children of different races when proceeding to late puberty and also explains why there were no significant differences in timing of sexual maturity among boys in NHES study. This finding of differences in the timing of sexual maturation in different communities suggested that normative reference data are required for different communities and settings.

The impact of environment on the timing of sexual maturation was highlighted by a

number of studies on a secular trend in pubertal timing from countries during economic transformations. These studies focused on females because the information on age at menarche is easy to obtain. A study in Poland showed that age at menarche in girls declined by 0.14 years in the rich region and 0.46 years in the poor region after the political and economic system transformation in 1989 (Łaska-Mierzejewska & Olszewska, 2004). Also, a retrospective study on women in South Korea who were born during economic transformations between 1920 and 1988, found the age at menarche in South Korean girls dropped by 4.1 years (Hwang *et al.*, 2003). Moreover, the decline in age at onset of puberty was also described in studies on foreign children adopted from developing countries to Western Europe (Proos *et al.*, 1991; Bourguignon *et al.*, 1992; Virdis *et al.*, 1998; Krstevska-Konstantinova *et al.*, 2001; Teilmann *et al.*, 2002). The improvement in socioeconomic and nutritional conditions by the transition from an underprivileged to a privileged environment were the main reasons provided to explain the decline in age at onset of puberty. More direct evidence for this hypotheses was provided by the difference in age at onset of sexual maturity between children living in privileged and underprivileged conditions in some developing countries from Asia, Africa and South-America where social economic inequality and the gap on living standards between urban and rural are prominent (Kulin *et al.*, 1982; Chompootaweep *et al.*, 1997; Macías‐Tomei *et al.*, 2000; Carrillo *et al.*, 2001; Campbell et al., 2004; Ma *et al.*, 2011; Natale & Rajagopalan, 2014). For example, in a study conducted in Kenya, in which pubertal growth and development were compared between in 342 privileged urban children and 347 impoverished rural children, found early stages of

sexual maturity were delayed 3 years in malnourish boys (Kulin *et al.*, 1982). Similarly, a study of pubertal timing in urban and rural boys in Zambia, reported the onset of puberty was delayed by 1.2 years in rural boys (Campbell *et al.*, 2004).

Recent studies showed an unexpected new decline in the age at onset of puberty among females during the past 15 years in developed countries, which was unlikely to have resulted from any major changes in socio-economic conditions (Sun *et al.*, 2002; Wu *et al.*, 2002; De Simone *et al.*, 2004; Castellino *et al.*, 2005; Aksglaede *et al.*, 2009). Here, it is thought that exposures to chemicals with endocrine-disrupting properties via food and environment may play a role. Many mechanistic experiments suggested that endocrine disrupting chemicals (EDCs) exhibits hormonal or anti-hormonal activity, usually oestrogenic or anti-androgenic activity (Damstra *et al.*, 2002; Sharpe & Irvine, 2004). EDCs can affect the HPG axis on receptor level or influence the synthesis and metabolism of hormones, receptors and transcription factors which can impact on sexual development and functioning (Takeyoshi *et al.*, 2002; Toppari, 2002; Rasier *et al.*, 2006). The effects of EDC's on pubertal development are difficult to investigate in epidemiological studies as it is difficult to separate it from the effects due to differences in nutritional status. Additionally, toxicological studies have shown that the effects of EDCs are highly dependent on the period of exposure, exposure dosage, duration of exposure, and half-life of the compounds.

2.4 PUBERTAL GROWTH SPURT

Adolescent growth spurt also known as pubertal growth spurt is one of the important markers of puberty. Pubertal growth spurt is characterized by significant somatic growth and measurable changes in body size and body composition. According to US National Center for Health Statistics data from cross-sectional growth studies, boys gain 30 cm to their final height during the pubertal growth which account for 17% of the final height (Abbassi, 1998). In general, pubertal growth consists of a phase of acceleration in growth rate in height until it reached the peak height velocity one of maturational milestone for mid-puberty, this is followed by a sharp decline and final stop in growth by the end of puberty. The stop in growth in height in late adolescence is the consequence of epiphyseal fusion, a sudden event in which growth cartilage is replaced by bone tissue (Rogol *et al.*, 2000). Besides growth in height, about 50% of the adult body weight is gained during puberty (Barnes, 1975; Marshall, 1978). In boys, peak weight velocity generally occurs in the same period of time as peak height velocity and then progresses at a gradually slower rate in a similar fashion to that of height velocity in the late puberty (Tanner, 1990).

The growth and development of children reflect the health of their population on a broad scale. Therefore, the reference standards for healthy growth is essential for local government and health organization to conduct a child health monitoring programme (Natale & Rajagopalan, 2014). Many national anthropometrical growth data have been published internationally (Ma *et al.*, 2001). Among all the growth reference data, the

CDC growth charts and the WHO growth references (Kuczmarski *et al.*, 2002) are the most popular growth standards for screening and monitoring of adequate growth in individuals and populations (de Onis & Habicht, 1996; Kuczmarski, *et al.*, 2000). Furthermore, the WHO growth references has been internationally used as a tool to assess the nutritional status of children and adolescents since the nutritional factor plays a vital role in supporting growth and development.

The relationship between nutritional status and growth of children and adolescents has been recognized a century ago. Already in the 1910s, Rietz (1906) showed the socio-economic impact on the height of school children in Berlin. He found privileged boys were at least 4-5 cm taller than those from low classes, and concluded that children from rich family's growth faster, are taller and mature earlier than those from poor families. Until now, there are still many cross-sectional studies investigate the growth of disadvantaged children in developing countries where the differences in nutritional status between rural and urban, and deprived and privileged remain substantial (Schwekendiek, 2009). For example, in Hungary, Eiben *et al* (2005) compared the growth and maturation of Hungarian urban and rural boys and girls in a national growth survey of 39,035 participants. They found on average that urban children had 0.29 higher height-for-age z-scores and 0.19 greater weight-for-age z-scores than their rural counterparts and this was statistically significantly different ($p<0.05$). Another cross-sectional study compared the height and weight of pre-school children between North and South Korea who started at the same economic level after the civil war took place

in 1960's. The results showed that pre-school children raised in the developed country of South Korea were 13 cm taller and 7 kg heavier than those who were raised in North Korea which still remained as a low-income country (Schwekendiek, 2009).

The abnormal growth of undernourished children referred to as stunting and wasting, is defined by the World Health Organization (WHO) as a value less than two standard deviations of the WHO Child Growth Standards median (WHO, 2014) for height/weight for age. In 2003, a cross-sectional study was also conducted on 1257 randomly selected children 10 to 15 years old from the North West Province in South Africa (Mukuddem-Petersen & Kruger, 2004). Using the CDC growth standards, it was found that stunting was mostly prevalent in rural boys (26.7%) compared to their urban counterpart (17.1%). Four years later, another cross-sectional growth survey was conducted among 3511 children and adolescents aged 1 to 20 who lives in Agincourt sub-district, Mpumalanga Province, one of the regions with the highest poverty rates in South Africa (Kimani-Murage *et al.*, 2010). Using 2006 WHO growth standards, it was found that about one in five children were stunted in the early life of development, and 12% of boys were stunted in the early puberty. More recently, in 2010, a cross-sectional study was conducted in South-Western Nigeria using the 2007 WHO reference (De Onis *et al.*, 2007) values to assess the growth of school adolescents from various socio-economic backgrounds. In this study, it was found that the height of adolescents from both urban and rural public schools were much more likely to be classified as stunted than those from urban private schools. Similarly, a study in China on children 9 to 20

years of age in 1989 to 2006 (Liu *et al.*, 2013), reported that urban children are approximately 40% less likely to be stunted (OR = 0.62; $p < 0.01$) or underweight (OR = 0.62; $p < 0.05$) in comparison to their rural counterparts using the 2006 WHO reference standards.

Despite the fact that nutritionally disadvantage children may tend to be shorter in height and lighter in weight during early puberty, late puberty may serves as a catch-up period to regain the growth losses (Dreizen *et al.*, 1967). In most of the growth studies on adolescents (Kulin *et al.*, 1982; Kimani-Murage *et al.*, 2010; Omigbodun *et al.*, 2010), the anthropometric disparities amongst children of different socio-economics backgrounds declined with increasing levels of maturation. For example, in the Mpumalanga cross-sectional growth survey (Kimani-Murage *et al.*, 2010), the prevalence of stunting was highest at Tanner Stage 1 for both girls and boys (an average of 9%), and then reduced to about 1% at Tanner stage 5. In the study in South-Western Nigeria, the prevalence of stunting in rural boys has fell from 33.4% at age of 14 to 18.6% at age of 19 (Omigbodun *et al.*, 2010). A full catch-up in height from rural boys in comparison to urban boys was demonstrated in a cross-sectional study conducted in Kenya (Kulin *et al.*, 1982). At the age of 10, the median height of urban boys were 12.5 cm taller than rural boys, and then rural boys reached a similar height to urban boys at the age of 18.

The knowledge of the mechanism of catch-up growth is very limited (Gafni *et al.*, 2001).

The catch-up in linear growth with maturational progression may due to food supplementation due to preferential feeding, public nutritional intervention programs, and/or being more independent for sourcing the food for themselves. As has been already demonstrated in animal experiments, catch-up growth is associated with refeeding after undernutrition (Osborne & Mendel., 1914; Bohman, 1955; Mitchell *et al.*, 1978). Whether the genetic potential for the final height will reached or not may depend on how great the growth insulting factor such as severity of food shortage, are. Severity of food shortage was identified by Graham *et al.* in a cross-sectional study on Peruvian children (Graham *et al.*, 1980) when they reported that urban boys caught-up more rapidly and had a higher final height than rural boys.

2.5 THE MALE REPRODUCTIVE SYSTEM

The hypothalamic–pituitary–gonadal axis (HPG) plays a crucial part in the development and regulation of the reproductive systems in males (World Health Organization, 2012) , page7). The HPG is activated during the first months of postnatal life (Andersson *et al.*, 1998; Burger, *et al.*, 1991; Forest, *et al.*, 1973), slows down during childhood, and then increase in activity at the onset of puberty (Lee, *et al.*, 1974). The reactivation of HPG during puberty will cause fluctuations in hormones levels resulting in variable downstream effects on the body. The hypothalamus produces gonadotropin-releasing hormone (GnRH) which stimulates pituitary gland to secrete gonadotropins such as luteinizing hormone (LH) and follicle–stimulating hormone (FSH), which regulate gonads on the production of sex steroids, predominately testosterone and estrogen. LH stimulates the production of testosterone by Leydig cell. FSH stimulates inhibin production in the testis, as well as activates Sertoli cells and induces the maturation of spermatocytes. Testosterone simulates the growth of male reproductive tissues and promotes secondary sexual characteristics, and estrogenic steroid hormones induce the maturation of the sperm and maintain a healthy libido. In males, oestrogen is converted from testosterone by aromatization. Sex hormone-binding globulin (SHBG) that binds to androgen and estrogen can also play a role in controlling these hormone levels. In male, approximately 97% - 98% of testosterone was bind by SHBG, therefore only 2% - 3% of the testosterone is free and biologically-active (Rosner, 1990).

2.6 REPRODUCTIVE HORMONES

As mentioned before, the activation of the HPG axis (figure 1) cause the onset of puberty which is characterised by fluctuations of gonadotropins and sex steroids levels. The right amount at the right time and in the right place of these reproductive hormones during puberty is essential to normal growth and secondary sexual characteristics development. FSH and LH are essential for human reproduction and lower secretions of these hormones can result in failure of gonadal function which leads to failure in the production of normal numbers of sperm in male. Oestrogen is known to have an important role in pubertal growth by controlling growth plate acceleration and fusion for both sexes (Perry *et al.*, 2008). Testosterone, in boys, promotes the most of the sexual maturational changes and is converted to oestrogen by aromatization. Clinical disorders of sex steroids synthesis and action will not only results in an abnormal of growth but also have irreversible negative impact on reproductive health (Perry *et al.*, 2008). There are few studies in the literature that have investigated the alternation of the reproductive hormones during the periods of pubertal development, and most of these studies were conducted in countries from Europe.

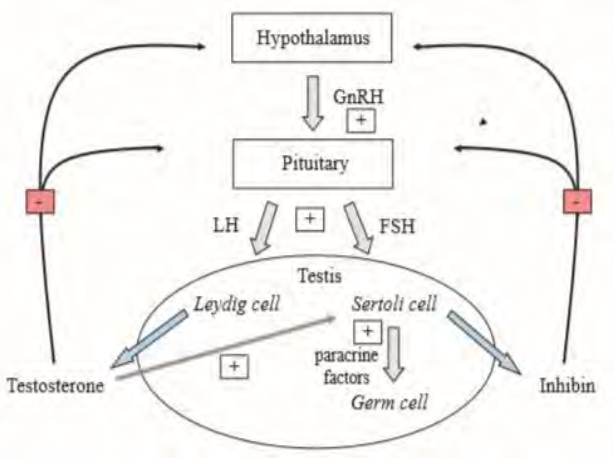

Figure 1 schematic representation of the hypothalamo-pituitary-testis axis (World Health Organization, 2012)

During puberty, all reproductive hormones increase from extremely low levels in pre-puberty to high levels at adulthood due to the activity of HPG axis. Previous studies on normal healthy boys from Europe (Andersson *et al.*, 1997; Chada *et al.*, 2003; Crofton *et al.*, 2002; Raivio *et al.*, 1998) all showed serum concentrations of reproductive hormones such as testosterone, estradiol and LH increase progressively throughout puberty. A cross-sectional study of 400 health Danish boys aged between 6 and 20 years old have reported that inhibin B, FSH, LH, testosterone and oestradiol levels at Tanner genital stage 2 (G2) were all significantly greater than those in late prepuberty. From stage G2, the inhibin B level was relatively constant, while the rest of the hormones continued to increase between stage G2 and stage G3. From stage G3, the FSH also reached a relatively constant level. The levels of LH, testosterone and oestradiol increased throughout of puberty (Andersson *et al.*, 1997). Similar overall patterns have been reported by others (Chada *et al.*, 2003; Crofton *et al.*, 1997; Raivio *et al.*, 1998). There are, however, variations in the timing and magnitude of the changes of hormonal serum levels by Tanner Stage. A longitudinal study of 195 Irish boys (Crofton *et al.*, 2002) showed that inhibin B reached a relatively constant level at stage G3 which is one stage later than the Danish study (Andersson *et al.*, 1997). In this study, the FSH level was increased throughout puberty. These findings were confirmed by a cross-sectional study of 78 boys from Czech Republic (Chada *et al.*, 2003). There are also

differences in the peak serum concentration of reproductive hormones during puberty, for example, in the Czechish boys (Chada *et al.*, 2003), the average peak concentration of Inhibin B, FSH, LH was 174.5 pg/mL, 2.3 UI/l and 3.17 UI/l respectively, in comparison to 224 pg/mL, 3.0 UI/l and 2.7 UI/l measured in a longitudinal study of 38 Finish boys (Raivio *et al.*, 1998) who used exactly the same methods for measuring the hormones. However, none of these differences were substantial and these variations in the magnitude of hormonal serum levels may be explained by the variations within each Tanner Stage as none of these studies have sample sizes more than a hundred for all pubertal stages.

2.7 REPRODUCTIVE HORMONES AS MARKERS FOR SEMEN QUALITY

According to Carlsen et al (1992), the sperm density has decreased significantly globally from 1938 to 1990. A meta-analysis (Swan *et al.*, 2000) of 101 studies on sperm density worldwide that included confounders in analysis, confirmed the decreasing trend in sperm density for 1938-1990, with approximately 1.5% decline per year in United States and approximately 3% decline per year in Europe and Australia. These findings of decreasing sperm quality worldwide have aroused particular public interest in semen quality of men in the general population (Carlsen *et al.*, 1992; Irvine *et al.*, 1996; Auger *et al.*, 1995). However, semen samples are difficult to obtain within sampling frames that allow comparisons due to the high levels of nonresponses, inappropriate sampling procedures, and intra-laboratory differences in methods (Kumanov *et al.*, 2006). Reproductive hormones are critical to spermatogenesis and maintenance of reproductive function in men (Meeker *et al.*, 2008) and in contrast to semen samples, blood samples are easier to obtain. Thus, a valid serum hormonal biomarker of spermatogenesis is of a particular interest for population studies.

FSH has been used as a marker of spermatogenesis in males at first. In the early 1990s, numerous reproductive health studies in male investigated the relationship between semen quality and pituitary hormones LH and FSH. The androgen, testosterone was used mainly in studies that focused on the men diagnosed with infertility. A study of 388 oligospermic men from an Infertility Clinic have showed that the mean FSH levels were 36.07 mIU/ml, 38.31 mIU/ml and 42.53 mIU/ml respectively in men with mild

(10-20 million sperms/mL), moderate (5-10 million sperms/mL) and severe oligospermia (1-5 million sperms/mL) and was significantly higher ($P<0.001$) in comparison to the 5.86 mIU/ml in non-oligospermic men (>20 million sperms/mL) (Subhan *et al.*, 1995). Similar the relationship of FSH levels between normal and oligospermic men was reported in previous studies (Abbaticchio *et al.*, 1990; Matzkin *et al.*, 1990; Hampl *et al.*, 1992). A study by Subhan et al (1995) reported FSH levels among men with severe oligospermia (1-5 million sperms/mL) was significantly ($P<0.01$) higher than in men with mild (10-20 million sperms/mL) or moderate (5-10 million sperms/mL) oligospermia (Subhan *et al.*, 1995). This suggest that FSH levels was also associated with severity of oligospermic condition (Bennet et al., 1991; Avril-Ducame *et al.*, 1990; Fauser *et al*., 1990). However, none of these studies have reported significant changes in the levels of LH and testosterone between oligospermic and normal individuals (Abbaticchio *et al.*, 1990; Matzkin *et al.*, 1990; Sharma *et al.*, 1992; Hampl *et al.*, 1992; Subhan *et al.*, 1995).

Later studies has suggested that serum inhibin B was a more direct serum marker of spermatogenesis compared to FSH. In the US, a clinical study of 145 normal couples with proven fertility showed that the levels of inhibin B in the men were significantly correlated with sperm concentration ($r=0.25$, $P=0.002$) and total sperm count ($r=0.20$, $P=0.02$) (Uhler *et al.*, 2003). Then, in a study of 75 male patients with infertility problems and 12 men with proven fertility found highly significant association between sperm count and inhibin B and FSH. They reported that total sperm count was

significant correlated with inhibin B (r=0.48, P<0.0001) and FSH levels (r=-0.41, P=0.0007). Furthermore, a study conducted on 174 men attending an infertility clinic found that inhibin B was significantly correlated (r=0.55) with sperm concentration (Anderson *et al.*, 1998c). Similarly, in a recent study of 55 fertile and 85 men presenting for infertility evaluations have reported that inhibin B levels correlated positively (R^2=0.27) and FSH correlated negatively (R^2=0.33) with total sperm concentration (Myers *et al.*, 2003). This finding suggesting the FSH and inhibin B are both good predictors of sperm concentration. Some epidemiologic studies has even been suggested that measuring the two hormones in serum could serve as a potential screening test for measuring semen quality. A study of 349 Danish men who had not previously attempted to achieve a pregnancy have reported that the predictive power of detecting oligospermia (sperm concentration below 20 mill/ml) among men who have inhibin B below 80 pg/ml and FSH above 10 IU/L, was 100% (the predict power for each hormone alone was 80.0% and 85.7% for inhibin B and FSH, respectively) (Jensen *et al.*, 1997). Additionally, a cross-sectional study of 47 malaria vector control workers exposed to DDT found a strong positive relationship (prevalence odds ratio (POR) = 37, CI:2-655) between high basal estradiol (> 50 pg/ml) and abnormal sperm morphology (proportion < 5%) and low sperm motility (proportion <50%). They also reported a significant positive relationship (POR = 8.2, CI:1.4-49.2) between low basal inhibin (<100 pg/ml) and low semen count (< 40 million) and density (< 20 million/ml), and weaker relationship between baseline FSH and low semen count and low density.

The reason for Inhibin B being a good marker of sperm quality may due the fact that is a product of Sertoli cells which determine both testicular size and sperm production (Orth *et al.*, 1988; Anderson & Sharpe, 2000). Only immature Sertoli cells proliferate, at around the onset of puberty when they undergo a series of changes in their morphology and function and finally switch from an immature proliferative state,to a mature, non-proliferative state. Previous studies indicated that Sertoli cells proliferate during two periods in fetal or neonatal life and in the peripubertal to early-pubertal period in human (Plant & Marshall, 2001). In the cohort study of Irish schoolchildren, inhibin B levels were found to be significantly ($P<0.01$) increased between 0-6 months and 6-12 months prior to pubertal onset (Crofton *et al.*, 2001) and reached to a relatively constant level in early puberty which is coincident with Sertoli cell proliferation. This finding suggested the inhibin B levels during puberty appear to reflect the Sertoli cell proliferation. Nuclear immunoexpression of the androgen receptor is another feature of mature Sertoli cells which is also commonly recognized as a late event in puberty (Plant and Marshall, 2001). This period coincides with a dramatic rise in the concentration of FSH and testosterone which might plays a role in the final maturation of the Sertoli cell at puberty. Based on these findings, the reproductive hormonal profile at puberty may predict the future reproductive health, particularly semen quality in adulthood.

2.8 RELATIONSHIP BETWEEN EACH PUBERTAL MEASUREMENT

As previously stated, puberty can be measured in different ways, such as sex characteristics, anthropometrical values, and reproductive hormones. In spite of the way these measurements to assess puberty are remarkably different from each other, they are more or less correlated.

Pubertal sexual development is clearly closely correlated with reproductive hormones. The alternation of reproductive hormones during puberty directly drives the sexual development as illustrated in the previous section.

Pubertal growth spurt is a maturational event, the growth depend highly on biological maturation. At a given chronological age, different timing or rate of biological maturation among adolescents can result in variant growth velocities in height and weight (De Onis & Habicht, 1996), and as a rule, early maturers tend to have higher dimensions in early puberty than late maturers (Prokopec & Lhotska, 1989). The biological maturation can be assessed by skeletal age and sexual maturity rating. In practice, sexual maturity rating is far more common to use than the skeletal age as the skeletal age assessment involves with radiography of the hand and wrist to calculate bone age (Mughal *et al.*, 2014). Studies on pubertal growth suggested that sexual maturity groups are more informative than chronological age groups on the changes in anthropometric parameters during puberty (Veldre & Jürimäe, 2004). Furthermore, the Expert Committee convened by the WHO recommended that maturational status should

be considered for interpreting anthropometric data for a better judgment of the normality of growth (Mughal *et al.*, 2014).

Sex steroids also play an important role in pubertal growth. It is believed that the pubertal growth spurt is predominately driven by increased secretion of sex steroids (Perry *et al.*, 2008). In both sexes oestrogen is the critical hormone for controlling growth plate acceleration and fusion, as well as exhibits an effect on the secretion of growth hormone. In boys, testosterone is important for growth of muscle and loss of fat in the limb (Tanner, 1990), as well as stimulate growth of bone through converting itself into oestrogen (Perry *et al.*, 2008).

.Each measurement of puberty may be an imperfect proxy for another, and none of these can represent a "gold standard" (Hayward, 2003). Thus, studying puberty by using multiple pubertal measurements can provide a comprehensive understanding on pubertal development. However, to our best knowledge, there is no any published works on pubertal study that combined all these measurements.

2.9 PUBERTAL HEALTH AMONG WESTERN CAPE RURAL BOYS

2.9.1 POOR NUTRITIONS

In most third world countries the nutritional condition of infants and children is very poor. This is certainly the case in most regions of Africa, home for an estimated 200 million undernourished people including both children and adults (Food and Agriculture Organization of the United Nations 2003). Despite South Africa being a middle-income country, undernutrition remains a problem with about one quarter of children under the age 6 is stunted by malnutrition (De Klerk et al., 2004). In 2005, 55% of households in South Africa had a monthly income below USD 100, and more than a third of children lived in households without access to clean drinking water. By 2013, according to the South African National Health and Nutrition Examination Survey (SANHANES) 26% of the population was facing hunger and a further 28% were at risk.

2.9.2 EXPOSURES TO ENDOCRINE DISRUPTING CHEMICALS

Western Cape Province is one of the fastest growing economies in South Africa, however the poverty and inequality still remarkable with a Gini index of 0.58 (Provincial Decision-Making Enabling, 2005). About 16.4 % of households were experienced hunger, and based on the fact that rural households earned far less income than urban households, the nutritional status of children raised in the rural area of the Western Cape is a major public health concern. Moreover, Western Cape Province is also struggled with low efficiency in nutrition promoting programs among children. For example, a year after the implementation of a national program of vitamin A supplementation aimed at children in 2002, over 70 percent of children in the Western

Cape were reported to not having received a supplement in the last half year (South African Medical Research Council 2003).

According to WHO, endocrine disruptors (EDCs) are substances that alter one or more functions of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or populations (Session, 2009). Many pesticides used in the rural Western Cape are EDCs (Dalvie *et al.*, 2009). Exposure to pesticides can occur via inhalation, ingestion, dermal contact, or across the placenta (Gilden *et al.*, 2010). It has been suggested that environmental EDCs are responsible for observed secular decreasing trend in sperm counts and quality and increasing incidence of reproductive tract abnormalities as well as testicular and prostate cancer in male during the past decades (Harrison *et al.*, 1997). Recent reviews (Cooper & Kavlock, 1997; Sharpe & Irvine, 2004; Fisher, 2004; WHO, 2009) concluded that the endocrine and reproductive effects of EDCs are believed to be due to their ability to act as oestrogen, anti-oestrogens, anti-androgens, steriodogenic enzyme inhibitors as well as interact with thyroid hormones and their receptors. A previous published cross-sectional study (English *et al.*, 2012) in the Western Cape rural area, showed that boys who living on farms had significantly lower levels of testosterone, LH, but higher in levels of estradiol and FSH in comparison to boys who resided in neighbouring not on farms during their pubertal development. The study also found smaller testes, shorter stature and lower body weight in farm boys compared with non-farm boys.

In Western Cape, agricultural products dominates the export industries contributing over 47% of all export commodities in 2010 (Wesgro, 2012). The agricultural sector uses the highest amount of pesticides in South Africa. Pesticides that have been associated with negative reproductive effects such as chlorpyrifos, an insecticide with weak estrogenic effect (Andersen *et al.*, 2002); endosulfan, an anti-androgenic insecticide (Andersen *et al.*, 2002); fenarimol, an anti-androgenic fungicide, are commonly used in Western Cape Province (English *et al.*, 2012). In previous surveys, pesticide endosulfan was detected in rural surface, ground water and even drinking water in the intensive farming areas of the Western Cape (Dalvie *et al.*, 2003).

During a critical period of development such as puberty, exposure to pesticides can result in increased risk for health outcomes (Gilden *et al.*, 2010). During the period that boys approach maturity and adulthood negative reproductive exposures may cause reproductive health problems (De Onis & Habicht, 1996).

2.9.3 LACK OF LOCAL REFERENCE DATA

There is a concern that even among healthy children the international clinical growth standards such as WHO and CDC growth standards may not be universally applicable across populations due to the variation of genetic factors and environmental factors (Ruff, 2002). On the other hand, the local pubertal growth reference data is extremely lacking in South Africa. For an example, in South African Demographic and Health Survey (SADHS 2002), one of the most recent national health survey, the

anthropometric references were only available for child under 5 years old and adolescents from age 15 to 19 years old, and did not cover other ages.

Considering the multitude of social impacts due to poverty and the fact that these populations are exposed to environmental impacts, there is a need for such local reference data (Dalvie *et al.*, 1999; English *et al.*, 2012).

2.10 CONCLUSION

There are a numbers of way in measuring pubertal characteristics such as the pubertal growth spurt, secondary sexual characteristics, and reproductive hormones. These measurements provide valuable tools in implementing adolescent's health monitoring programmes.

Previous research has found that the timing of sexual maturation, and the pattern of anthropometric growth and hormonal changes are under the influence of genetic and environmental factors, such as ethnic group, socio-economic status, nutritional availability and geographic location, as well as environmental exposures such as exposure to endocrine disrupting chemicals. The global variations in these factors has posed a great challenge in the generalizability of international references, particularly, between developed and developing countries. The knowledge of pubertal development in the rural area of the Western Cape rural is lacking.

A study on pubertal growth and reproductive development among boys in the rural Western Cape of South Africa that experience a multitude of socio-economic factors such poverty and malnutrition as well environmental exposures will increase the scientific knowledge internationally in this area. The knowledge gained from such a study could be considered for future children health monitoring programme and health management strategies in the Western Cape rural area.

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PART C: MANUSCRIPT

Anthropometric measurements, serum reproductive hormonal levels and sexual development among boys in the rural Western Cape

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ABSTRACT

Background

The timing of growth and sexual maturation during puberty may differ in different populations due to differences in genetic, socio-economic and environmental factors. There is currently no data on growth and sexual maturation of rural Western Cape boys in South Africa.

Methods

A cross-sectional study of 269 school boys was conducted in rural Western Cape in South Africa. Testing included measurement of serum lutenizing hormone (LH), follicle stimulating hormone (FSH), testosterone, sex hormone binding globulin (SHBG) and estradiol (E2); height, weight and BMI measurements; assessment of sexual maturity (using Tanner Stages) and a questionnaire (demographics and general medical history).

Results

The median age of Western Cape rural boys at pubertal onset (Tanner Stage 2) and Tanner Stage 5 was 11.6 and 14.7 years, respectively. More than 25% and 50% of height and weight measurements were below the 25^{th} and 50^{th} percentile respectively of the CDC and WHO growth references at Tanner stage 1-3 but not at higher Tanner Stages. After adjusting for confounders, serum FSH and LH increased significantly ($P<0.05$) from Tanner Stage 1 to 4 and then plateaued. Serum testosterone and estradiol started to increase significantly ($P<0.05$) only after Tanner stage 2.

Conclusion

The age at onset of puberty and early anthropometric development but not end of puberty and late anthropometric development of Western Cape rural boys were delayed when compared to populations from other settings indicating catch-up in pubertal development at higher Tanner Stages. These delays in pubertal development are possibly due to nutritional, socio-economic and environmental exposures experienced by these boys. Changes in serum levels of LH, FSH, testosterone and oestradiol in boys from the rural Western Cape were consistent with other populations. Futhermore, serum FSH levels, which have been found to be negatively associated with semen quality in men, were relatively high in Western Cape rural boys when late puberty was reached. Initiatives to improve nutrition amongst Western Cape rural communities.

INTRODUCTION

Puberty involves a number of processes including the adolescent growth spurt, rapid changes in body composition, development of secondary sex characteristics, activation of hypothalamic–pituitary–gonadal axis activity, achievement of fertility, and behavioral and psychological changes. Physical development during puberty can be measured by assessing secondary sex characteristics, bone age, growth spurt, or hormonal levels with each measurement capturing a different aspect of the pubertal process and none of these representing the "gold-standard" (Hayward, 2003).

The CDC and the WHO Growth standards are the most well-known growth standards for anthropometric measurements (height, weight, body mass index), and are used internationally to monitor growth and identify potential health- or nutrition-related problems among children and adolescents (Grummer-Strawn et al., 2010). The growth of nutritionally disadvantaged children are generally characterized by a short statue and low weight, as well as late biological maturation (Kulin *et al.*, 1982; Eiben *et al.*, 2005; Kimani-Murage *et al.*, 2010). On the other hand, pubertal growth not only determine the final height in the adulthood, but also may serve as a catch-up period to regain the previous growth loss (Graham *et al.*, 1980; Williams, 1981; Kulin *et al.*, 1982; Campbell *et al.*, 2004). It is therefore important to have local growth reference ranges by sexual maturation from developing regions.

The development of secondary sex characteristics is driven by the activity of reproductive hormones in the hypothalamic–pituitary–gonadal axis (HPG) and is commonly measured by five Tanner Stages (Tanner, 1962). The HPG axis which is the major regulator of the mature reproductive system, is quiescent in childhood and then reactivated at the onset of puberty (Lee *et al.*, 1974). This

reactivation of the HPG axis is characterized by a sudden increase in the concentration of gonadotropins (follicle–stimulating hormone (FSH) and luteinizing hormone (LH) stimulated by gonado-trophin-releasing hormone (GnRH) in the pituitary gland, and sex steroids (testosterone and oestradiol) stimulated by gonadotropins in the gonads (Andersson *et al.*, 1997).

FSH and inhibin B released by Sertoli cells after puberty and which functions as a negative feedback on FSH secretion, are useful markers of spermatogenesis and Sertoli cell function reflecting the quality of semen, particularly semen concentration (Meeker *et al.*, 2007; Uhler *et al.*, 2003; Mabeck *et al.*, 2005). A Danish study of 430 couples with unknown fertility found 100% predictive power for detecting sperm counts below 20 mill/mL by using the criteria of basal inhibin < 80 pg/ml and FSH > 10 miu/ml (Jensen *et al.*, 1997). Besides inhibin B and FSH, a recent cross-sectional study of 47 DDT exposed malaria vector control workers reported a positive relationships (Odds Ratio = 37, CI: 2-655) between high basal estradiol (> 50 pg/ml) and abnormal semen morphology (proportion < 5%) and low motility (proportion <50%) (Dalvie & Myers, 2005).

Puberty is a critical development period for the establishment of mature reproductive system as well as the achievement of adulthood levels of reproductive hormones. Therefore, monitoring the change of reproductive hormones during puberty may provide an indication of reproductive development in boys and their fertility later in life.

Data on anthropometric characteristics, reproductive hormones and sexual development in pubertal children in the rural Western Cape of South Africa have, to our knowledge, not been published before. Considering the multitude of social impacts due to poverty and as well as environmental impacts, there is a need for such local reference data among these populations. There are also few

studies available in the literature and none in South Africa that have investigated the changes in serum reproductive hormone levels during pubertal development of children (Andersson *et al.*, 1997; Raivio *et al.*, 1998; Crofton *et al.*, 2002; Chada *et al.*, 2003).

In this analysis, we present cross-sectional descriptive data on anthropometric characteristics, secondary sexual characteristics, testicular volumes and reproductive hormones from boys residing in the rural Western Cape in South Africa and participating in a study that have investigated the effects of pesticide exposure on these populations. Additionally, the relationship between these parameters are investigated.

METHODS

Study design, population and sampling

Data was gathered from a cross-sectional study of Western Cape rural boys in South Africa which was conducted between April 2007 to March 2008, was used in this analysis. The sampling for the study has been described in detail elsewhere (English *et al*, 2012). Briefly, the eight most accessible primary and secondary schools from three agricultural areas (Hex River Valley, Grabouw, Piketberg) and their neighboring non-agricultural rural areas in the Western Cape Province were chosen for the study population. School boys aged from 5 to 19 years were chosen in order to cover the full age range for pubertal development.

Four hundred and ninety-two boys' parents consented to participate in the study and were stratified according to whether they had lived on a farm or not at the time of the study and by area. Then 274 boys were selected including all boys (n = 180) living on a farm and 94 not living on a farms. The former group was chosen by random systematic sampling, stratified equally by age groups. A further 5 boys all who lived on a farm did not participate in the study leaving only 269 participants. In the study sample, 15.2% were between 5 to 9 years old (pre-pubertal), 28.6% were between 9.1 to 11 years old (early-puberty), 44.2% were between 11.1 to 14 years old (mid-puberty) and 12% were older than 14 years of age (post-puberty).

Measurements

A questionnaires was administered to a parent of the participant by trained fieldworkers using mobile technology. The questionnaire was developed by the study team, led by the principal investigator, and was based on previous local studies in similar populations (Dalvie *et al*., 1999;

Dalvie *et al.*, 2004). The questionnaire included sections on demography, genital health history, and general medical history.

Anthropometric measurements and sexual maturity assessment was conducted by a male nurse who was trained by a local reproductive health specialist. Training was conducted over a period of 2 days, mainly focusing on demonstrating how to perform anthropometric measurements, use of the orchidometer to measure testicular volume and assessing the sexual maturity rating (SMR) by examination of boy patients and using visual material.

Height and weight were recorded, using a calibrated scale, according to standardized methods, and body mass index (BMI) was calculated. The Sexual Maturation Rating was recorded according to 5 Tanner Stages (Tanner & Whitehouse, 1976) Testicular volume was assessed using a standardized set of wooden testicular beads (Zachmann *et al*., 1974) and further examinations were conducted to assess for the presence of genital scars, penis and testicular abnormalities such as congenital hydroceles, undescended testes, congenital inguinal hernias and hypospadias.

Blood samples (12 ml) were collected between morning and early-afternoon. The blood samples were centrifuged at 5000 revolutions per minute in the field and the serum stored and transported on ice to National Health Laboratory Sciences (NHLS) facility at Groote Schuur Hospital in Cape Town within 24 hours. LH, FSH, testosterone, oestradiol, and SHBG were measured using electrochemiluminescence immunoassays (ECLIA) on a Roche Cobas Modular E170 analyzer.

This study was done according to the Declaration of Helsinki of the 25th World Medical Assembly (World Medical Association, 2001). The research proposal of the study was approved by the

University of Cape Town's Research Ethics Committee (HREC REF 943/2014). The study respected participants' autonomy, the right to the information and confidentiality of their information.

Statistical analysis

Data analysis was performed using Stata 12 statistical software (StataCorp, Texas, USA). Exploratory data analysis was conducted first. Each variable was examined for missing data, and then assessed by univariate graphs and summary statistics for any potential outliers. Normality for continuous variables was assessed using histograms and Shapiro-Wilk tests. Descriptive statistics were calculated and expressed as medians and interquartile intervals.

To compare with CDC growth charts (Centre for Disease Control and Prevention, 2009) and WHO growth reference (World Health Organizations, 2015), two binary variables for each variable such as height, weight and BMI were generated by using with the 25^{th} and 50^{th} percentile ($\leq 25^{th}$ and 50^{th} percentile = 1, $\geq 25^{th}$ and 50^{th} percentile = 0) for age from both growth references as cut-offs.

Bivariate analysis was conducted to explore the relationships between independent variables (age, anthropometric variables, testicular volume and hormones) and possible predictors to identify potential counfounders. Household income, birth weight and SHBG were tested by Pearson's correlation test (for normally distributed data) and Spearman's Rank correlation test (for non-normally distributed data), and residential location, general health, disease history and testicular

related problems were tested by t-test (for normally distributed data) and Wilcoxon rank sum test (for non-normally distributed data) with all predictor variables. Confounders were selected for multivariate model building if the association was $p < 0.1$. Linear regression analysis was used for investigating the multivariate association between dependent and predictor variables by Tanner Stage (Dummy variables were generated from the 5 discrete Tanner Stage including Tanner Stage 1 vs Tanner Stage2, Tanner Stage 2 vs Tanner Stage3, Tanner Stage 3 vs Tanner Stage 4; Tanner Stage 4 vs Tanner Stage 5) Model building was started with an empty model containing only the constant and then adding predictors. Forward Stepwise Regression procedure was used to select the best combination of confounders. The likelihood ratio test was used to determine whether the model has been significantly improved by adding each confounder. The variable combination with lowest Aikake's Information Criterion (AIC) statistic was selected for the next step until the accomplishment of the best combination of variables and then the key independent variable to the final model. Regression diagnostics were determined by the goodness of fit of the model, and outliers or influential observations were assessed.

RESULTS

Participation, demographic characteristics, socioeconomics information and health status and medical history

One hundred and seventy seven of the participating boys (65.8%) lived or previously lived on a farm and 92 boys (34.2%) lived only in a town.

Overall the median age of the participants was 11.6 years (Table 1) and the median birthweight was 2.9 kilograms (n = 233). The median household income (n = 262) was R 2000.

The sexual maturity assessment of the boys found that (Table 1) 39.78% (n=107) of boys were classified in the pre-pubertal stage, 43.5% (n=117) in the mid-puberty stage (Tanner Stage 2 and 3), and 16.35% (n=44) in late puberty (Tanner stage 4 and 5).

A small proportion of parents (3.7%) reported that their participating children were in a poor health condition. Lifetime tuberculosis and asthma reported was more than 5%, about and a third (29.3%) previously had mumps. About 5% of participants were born with abnormal testis and less than 3% had a previous testicular injury or operation or disease.

Table.1 Participation, demographic characteristics, socioeconomics information

Variables	**Median (IQR)**
Age (years), (n=269)	11.58 (9.42; 13.17)
Household Income (Rands), (n=262)	2000 (1300;2720)
Birth weight (kilogram), (n=233)	2.9 (2.51;3.3)
Variables	**N (%)**
Lifetime Residential Location	
Farm	177 (65.80%)
Non-farm	92 (34.20%)
Number of Boys within each Tanner Stage	
Tanner Stage 1	107 (39.78%)
Tanner Stage 2	78 (29.00%)
Tanner Stage 3	39 (14.50%)
Tanner Stage 4	36 (13.38%)
Tanner Stage 5	8 (2.97%)
General Health	
Good to Excellent	259 (96.3%)
Poor	10 (3.7%)
Medical History (Lifetime)	
Diabetes	2 (0.7%)
Tuberculosis	15 (5.6%)
Epilepsy	3 (1.1%)
Asthma	25 (9.3%)
Heart Problem	2 (0.7%)
HIV	1 (0.4%)
Foetal Alcohol Syndrome	2 (0.7%)
Mumps	79 (29.3%)
Pesticide poisoning	2 (0.7%)
Testicular Related Problems	
Born with abnormal testes	12 (4.5%)
Previous testicular injury	8 (3.0%)
Previous testicular operation	5 (1.9%)
Reported testicular disease	7 (2.6%)

Testicular development and age of onset of sex maturational development

Age and testicular volumes were significantly (P<0.05) positively associated with the increase in Tanner Stage until Tanner stage 4 (Table 2).

Table.2 Mean and interquartile range of age, testicular volume, anthropometric variables and reproductive hormone levels per Tanner Stage amongst boys in the rural Western Cape

TANNER STAGE VARIABLE	TANNER STAGE 1	TANNER STAGE 2	TANNER STAGE 3	TANNER STAGE 4	TANNER STAGE 5
AGE (YEAR)	9.3 (8.8; 10.7)	11.7* (10.4; 12.8)	13.1* (12.5; 13.9)	13.9* (13.0; 15.0)	14.7 (12.9; 15.5)
TESTICULAR VOLUME (ML)	2.5 (2.5-3.5)	5.5* (4.0-9.0)	15.0* (11.0-17.5)	20.0* (17.5-22.5)	22.5 (17.5-22.5)
ANTHROPOMETRIC VARIABLES					
HEIGHT (CM)	128.5 (124.0-135.0)	137.2* (131.2-141.5)	149.7* (144.4-159.0)	162.0* (153.8-166.5)	165.5 (165.1-174.0)
WEIGHT (KG)	27.0 (24.0-32.0)	32.0* (29.0-37.0)	42.0* (39.0-48.0)	49.0* (45.0-54.5)	57.0* (51.5-64.0)
BMI (KG^2/CM)	16.64 (15.23-18.12)	17.25 (15.55-18.30)	18.50* (17.23-22.21)	18.77 (17.76-20.64)	20.90 (19.17-23.44)
REPRODUCTIVE HORMORNES					
FSH (IU/L)	0.9 (0.5-1.5)	1.9* (1.3-3.1)	3.1* (2.3-4.9)	5.0* (3.2-7.5)	3.4 (3.2-7.0)
LH (IU/L)	0.05 (0.05-0.2)	0.7* (0.2-1.0)	1.65* (1.3-2.1)	3.0* (1.8-4.2)	2.8 (1.9-3.8)
TESTOSTERONE (NMOL/L)	0.05 (0.05-0.1)	0.2 (0.05-0.6)	3.9* (1.6-8.4)	10.45* (5.4-13.6)	12.9* (10-18.9)
OESTRADIOL (PMOL/L)	31.5 (23.1-45.5)	40.45 (29.7-50.4)	56.5* (46.1-72.2)	79.35* (61.5-88.5)	100.9* (87.2-115.2)
SHBG (NMOL/L)	121.8 (92.9-145.6)	104.8* (86.7-129.6)	67.5* (54-78.7)	44.9* (37.6-50.2)	46.2 (36.6-55.2)

*P<0.05 compared to the previous Tanner Stage (Linear Regression Analysis)

Relationship between anthropometric variables and sexual development

A description of anthropometric characteristics, sexual development and reproductive hormone levels have been presented in English et.al (2012) and are presented in Table 2 by Tanner Stage. All anthropometric variables increased during the development of puberty.

Multivariate analysis between Tanner Stage and anthropometric variables in Table 3 shows that height and weight all increased significantly ($P < 0.05$) by Tanner Stage but height did not increase significantly between Tanner stage 4 and Tanner stage 5. The only significant increase in BMI was between Tanner Stage 2 and 3, with the regression coefficient projecting an increase of 2.27 kg^2/cm (95% confident interval between 1.13 and 3.414 kg^2/cm). Coincidently, the most significantly growth in height and weight was also found in between Tanner Stage 2 and 3, with the regression coefficient projecting an increase of 13.8 cm in height, and 11.9 kg increase in weight. This indicated that the most rapid period of pubertal growth lies between Tanner stage 2 and stage 3 which corresponds to an age of 11.6 to 13.1 years among the Western Cape rural boys.

Table 3. Multivariate association between anthropometric variables and tanner stage for boys from the rural Western Cape after adjusting for lived on farm or not, family income, birth weight, general health condition, prenatal exposures, and testicular related problems

Outcomes	n	p-value	B-coefficient	95% confidence interval
Height				
Tanner stage 2 vs stage 1	185	<0.001	7.536	(4.871; 10.201)
Tanner stage 3 vs stage 2	117	<0.001	13.804	(10.122; 17.486)
Tanner stage 4 vs stage 3	75	<0.001	8.576	(3.931; 13.222)
Tanner stage 5 vs stage 4	44	0.296	3.725	(-3.280; 10.729)
Weight				
Tanner stage 2 vs stage 1	185	<0.001	3.956	(1.796; 6.115)
Tanner stage 3 vs stage 2	117	<0.001	11.911	(8.927; 14.895)
Tanner stage 4 vs stage 3	75	0.014	4.737	(0.972; 8.502)
Tanner stage 5 vs stage 4	44	0.012	7.295	(1.618; 12.972)
BMI				
Tanner stage 2 vs stage 1	185	0.446	0.321	(-0.507; 1.149)
Tanner stage 3 vs stage 2	117	<0.001	2.270	(1.126; 3.414)
Tanner stage 4 vs stage 3	75	0.706	-0.276	(-1.720; 1.167)
Tanner stage 5 vs stage 4	44	0.109	1.775	(-0.401; 3.951)

When comparing the age specific height and weight with CDC growth charts and WHO growth standards more than 25% and 50% of Western Cape rural boys were below the 25th and 50th percentile respectively of both WHO and CDC growth charts during the pre- and early-puberty (Table 4).After early puberty (Tanner Stage 3), the height and weight measurements caught up with CDC and WHO standards. Slightly less boys were below WHO charts than CDC charts.

Table 4. Comparison of anthropometric measurements with CDC and WHO charts

Height, weight and BMI < 25th & 50th percentile over each Tanner Stage	**vs CDC growth chart n (%)**	**vs WHO growth chart n (%)**
Body Mass Index,		
≤50th percentile for age	**138 (51.49%)**	**116 (43.28%)**
Tanner Stage 1	54 (50.47%)	44 (41.12%)
Tanner Stage 2	46 (58.97%)	42 (53.85%)
Tanner Stage 3	16 (41.03%)	14 (35.90%)
Tanner Stage 4	19 (52.78%)	13 (36.11%)
Tanner Stage 5	3 (37.50%)	3 (37.50%)
≤25th percentile for age	**74 (27.61%)**	**64 (23.88%)**
Tanner Stage 1	32 (29.91%)	29 (27.10%)
Tanner Stage 2	27 (34.62%)	24 (30.77%)
Tanner Stage 3	7 (17.95%)	6 (15.38%)
Tanner Stage 4	7 (19.44%)	5 (13.89%)
Tanner Stage 5	1 (12.50%)	0 (0.00%)
Height,		
≤50th percentile for age	**194 (72.39%)**	**179 (66.30%)**
Tanner Stage 1	83 (77.57%)	76 (70.37%)
Tanner Stage 2	64 (82.05%)	60 (76.92%)
Tanner Stage 3	25 (64.10%)	22 (56.41%)
Tanner Stage 4	17 (47.22%)	16 (44.44%)
Tanner Stage 5	5 (62.50%)	4 (50.00%)
≤25th percentile for age	**152 (56.72%)**	**136 (50.37%)**
Tanner Stage 1	66 (61.68%)	59 (54.63%)
Tanner Stage 2	54 (69.23%)	47 (60.26%)
Tanner Stage 3	19 (48.72%)	17 (43.59%)
Tanner Stage 4	10 (27.78%)	9 (25.00%)
Tanner Stage 5	3 (37.50%)	3 (37.50%)
Weight,		
≤50th percentile for age	**179 (66.54%)**	
Tanner Stage 1	74 (69.16%)	
Tanner Stage 2	65 (83.33%)	
Tanner Stage 3	22 (56.41%)	
Tanner Stage 4	15 (41.67%)	
Tanner Stage 5	3 (37.50%)	
≤25th percentile for age	**112 (41.64%)**	
Tanner Stage 1	50 (46.73%)	
Tanner Stage 2	43 (55.13%)	
Tanner Stage 3	11 (28.21%)	
Tanner Stage 4	8 (22.22%)	
Tanner Stage 5	0 (0.00%)	

Serum Reproductive hormones by Tanner Stage

The multivariate relationship between Tanner Stage and reproductive hormones are shown in Table 5. Serum FSH and LH increased significantly ($P<0.001$) from Tanner Stage 1 to Tanner Stage 4, and then reduced non-significantly until the end of puberty. Between Tanner Stage 1 to Tanner Stage 4, each increase in the Tanner Stage was associated with 1.034 IU/L to 1.821 UI/L increase in the serum levels of FSH, and 0.545 to 1.163 IU/L increase in LH. Serum levels of testosterone and oestradiol was increased non-significantly from Tanner Stage 1 to Tanner Stage 2, and then increased significantly ($P<0.05$) thereafter. The increase in levels of testosterone and oestradiol were highest during Tanner Stage 4 and 5. In contrast, the serum levels of SHBG started decreasing significantly at the onset of puberty until Tanner stage 4, and then remained constant to the end of puberty. When adjusting for SHBG, there were no significant increases in serum levels of LH and FSH during puberty (results not shown).

Table 5. Multivariate Linear Regression relationship between Tanner Stage and reproductive hormone levels for boys in the rural Western Cape after adjusting for lived on farm or not, family income, general health condition, and ever had any testicular related problems

Outcomes	n	P-value	B-coefficient	95% confidence interval
FSH				
Tanner Stage 2 vs Stage 1	170	<0.001	1.034	(0.554; 1.514)
Tanner Stage 3 vs Stage 2	110	<0.001	1.416	(0.765; 2.067)
Tanner Stage 4 vs Stage 3	72	<0.001	1.821	(0.986; 2.657)
Tanner Stage 5 vs Stage 4	41	0.287	-0.697	(-1.985; 0.590)
LH				
Tanner Stage 2 vs Stage 1	172	<0.001	0.545	(0.309; 0.781)
Tanner Stage 3 vs Stage 2	111	<0.001	1.163	(0.843; 1.484)
Tanner Stage 4 vs Stage 3	71	<0.001	0.963	(0.545; 1.380)
Tanner Stage 5 vs Stage 4	40	0.805	0.080	(-0.558; 0.718)
Oestradiol				
Tanner Stage 2 vs Stage 1	173	0.07	6.120	(-0.494; 12.733)
Tanner Stage 3 vs Stage 2	112	<0.001	19.993	(10.968; 29.018)
Tanner Stage 4 vs Stage 3	72	0.022	13.621	(2.011; 25.230)
Tanner Stage 5 vs Stage 4	41	0.006	25.320	(7.430; 43.210)
Testosterone				
Tanner Stage 2 vs Stage 1	174	0.186	0.548	(-0.266; 1.363)
Tanner Stage 3 vs Stage 2	111	<0.001	5.237	(4.127; 6.347)
Tanner Stage 4 vs Stage 3	72	<0.001	3.347	(1.922; 4.772)
Tanner Stage 5 vs Stage 4	41	<0.001	4.621	(2.425; 6.818)
SHBG				
Tanner Stage 2 vs Stage 1	172	0.008	-13.531	(-23.454; -3.608)
Tanner Stage 3 vs Stage 2	111	<0.001	-38.669	(-52.146; -25.191)
Tanner Stage 4 vs Stage 3	71	0.009	-23.341	(-40.904; -5.778)
Tanner Stage 5 vs Stage 4	40	0.999	0.023	(-26.805; 26.850)

DISCUSSION

This study found that the median age at the onset of puberty (Tanner stage 2) of Western Cape rural boys to be 11.5 years which was delayed in comparison to boys from settings with higher socio-economic status such as African American boys (9.5 years old) and American Caucasian boys (10.1 years old) from a US national probabilistic sampling study conducted between 1988 and 1994 (Sun *et al*., 2002); urban African boys (10.4 years old) and caucasian boys (9.8 years old) from a birth cohort in Soweto, Johannesburg, South Africa (Jones *et al*., 2009) in 2004; urban boys from Nairobi, Kenya (9.7 years old) in 1980 (Kulin *et al*., 1982) and urban boys whose parents were employed in the formal sector from Choma, Zambia (11.2 years old), in 1993 (Campbell *et al*., 2004). The late onset of puberty in Western Cape rural boys compared to African boys from developed countries and urban settings may be due to a lower nutritional status resulting from a lower socio-economic status of rural Western Cape boys. The study conducted in Zambia used a testicular volume of 3 mL as the cut-off value for onset of puberty in boys (Campbell *et al*., 2004), while the rest of the studies (Sun *et al*., 2002; Jones *et al*., 2009) used Tanner Genital Stage 2 as the onset of puberty.

Despite the delay in the age at onset of puberty, the median age at Tanner Stage 4 and 5 (13.9 and 14.7 years respectively) in Western Cape rural boys was similar to those of boys in Kenya and African American boys (Sun *et al*., 2002). This may suggest that the duration of puberty in Western Cape rural boys is shorter than those of African populations with higher socio-economic status resulting from a catch-up in pubertal development similar and may be related to the catch-up in the anthropometric development as will be discussed in a later section. It should be noted that these

results on the duration of pubertal development could have been affected by the smaller sample of boys over the age of 14 years in the study (12%) compared to the younger participants.

The low anthropometric readings of Western Cape rural boys when compared to CDC and WHO growth standards before Tanner Stage 3 and subsequent catch-up during Tanner Stages 4 and 5 could likely be attributed to poor nutritional conditions and adverse exposures also identified in other populations with stunting ((Kulin *et al*., 1982; Marshall & Tanner, 1986, Kimani-Murage *et al*., 2010). Puberty was identified as a catch-up period to make up the growth losses in animal experiments on re-feeding after undernutrition (Osborne & Mendel., 1914; Bohman, 1955; Mitchell *et al*., 1978). In epidemiologic studies on impoverished children when compared to privileged children for both sexes (Kulin *et al*., 1982; Prader *et al*., 1963; Campbell *et al*., 2004), catch-up growth in children that mature later have been attributed to improved nutrition due to preferential feeding in these children and due to the fact that these children being more independent in sourcing food (Kulin *et al*., 1982; Gillett-Netting *et al*., 2004).

This study found that LH and FSH in Western Cape rural boys increased substantially during Tanner Stages 1-4 and oestradiol and testosterone increased between Tanner Stages 2-5. Tables 6 and 7 compare the levels of gonadotropins and sex steroids in this study to other published studies which are all from European countries (Andersson, *et al*., 1997; Raivio, *et al*., 1998; Crofton, *et al*., 2002; Chada, *et al*., 2003) as no data could be found from other settings. It should also be noted that two of the previous studies (Raivio, *et al*., 1998; Crofton, *et al*., 2002) are longitudinal studies while the other two are cross-sectional. For both LH and FSH the pattern of increase and the levels of

hormones are similar to those of European boys apart from the fact that in Danish boys (Anderson *et al*., 1997) FSH increase until Tanner 3 and then stabilizes. The FSH levels at Tanner Stage 4 in the current study are also higher than those in previous studies. For testosterone, the levels are lower but the patterns of increase are similar to that of European boys. The generally high levels of testosterone in the study by Anderson *et al* (1997) might be a reason for the stabilization of FSH levels at Tanner Stage 3 resulting from negative feedback on the hypothalamous that lead to the lowering of GnRH and gonotrophins levels. The low levels of testosterone among Western Cape rural boys especially during Tanner Stage 1 and Tanner Stage 2 when compared to the European studies might have played a role in the delays in early puberty as testosterone stimulates secondary sexual characteristics in males. Only one previous study (Andersen *et al.*, 1997) had investigated oestradiol in which the pattern of increase is similar to this study but the levels of oestradiol are lower.

Serum FSH was found to be negatively associated with semen quality in men in previous studies and also a marker of semen quality in men (Jensen *et al.*, 1997; Meeker *et al.*, 2007). Serum FSH and inhibin B was also found to be associated with the number of Sertoli cells which determine both testicular size and daily sperm production in men (Sharpe *et al.*, 2003). During puberty immature proliferating Sertoli cells switches to mature non-proliferating Sertoli cells which determines the final number of Sertoli cells in adulthood (Sharpe *et al*., 2003; Griswold, 1998) and this could explain the stabilization of the testicular volume at pubertal stage 4. FSH levels at Tanner Stage 4 in boys from the rural Western Cape in comparison to those in European boys listed in Table 6 might therefore suggest low sperm quality during adulthood amongst these communities.

Table 6. Levels of gonadotropins during sexual development in this study compared with other published studies

Study	Country	Study Type		FSH					LH				
				Tanner Stage 1	Tanner Stage 2	Tanner Stage 3	Tanner Stage 4	Tanner Stage 5	Tanner Stage 1	Tanner Stage 2	Tanner Stage 3	Tanner Stage 4	Tanner Stage 5
Current study	South Africa	C-S	Median IQR N	**0.9** 0.5-1.5 98	**1.9*** 1.3-3.1 72	**3.1*** 2.3-4.9 38	**5.0*** 3.2-7.5 34	**3.4** 3.2-7.0 7	**0.05** 0.05-0.2 99	**0.7*** 0.2-1.0 73	**1.65*** 1.3-2.1 38	**3.0*** 1.8-4.2 33	**2.8** 1.9-3.8 7
A.M. Andersson *et al.*, 1997	Danish	C-S	Median 90% PI N	**0.85** 0.25-2.55 154	**1.95*** 0.07-4.39 47	**3.50*** 0.94-9.68 27	**3.61** 1.98-6.88 31	**3.10** 1.38-7.52 128	**0.08** 0.05-0.99 154	**0.88*** 0.11-2.97 47	**2.03*** 0.51-5.42 26	**2.89*** 1.11-5.89 31	**3.40*** 1.53-6.33 129
T. Raivio *et al.*, 1998	Finland	L	Mean ±SD N	**1.8** 1.2-2.6 16	**1.8** 1.2-2.8 37	**1.9** 1.2-3.1 37	**2.9** 1.7-4.9 37	**3.0** 1.8-4.9	**0.3** 0.1-0.8 16	**1.1** 0.5-2.3 37	**1.4** 0.8-2.5 37	**2.0** 1.3-3.0 37	**2.7** 1.6-4.5
P. M. Crofton *et al.*, 2001	Ireland	L	Median IQR N	**1.2** 1.0-2.1 90	**2.2** 1.6-2.9 38	**2.7*** 1.9-3.3 39	**3.5** 2.7-5.0 18	**4.2** 3.7-5.1 10					
M. CHADA *et al.*, 2002	Czech Republic	C-S	Mean Min~Max N	**0.42** 0.20-0.58 11	**0.85** 0.21-1.48 8	**1.46** 0.49-2.63 12	**2.30** 0.93-3.65 10		**0.21** 0.10-0.44 11	**0.61** 0.12-1.43 8	**1.23** 0.45-2.39 12	**3.17** 1.05-5.54 10	

L: longitudinal; C-S: cross-sectional; IQR: interquartile range; N: number of boys; PI: predict interval; SD: standard deviation;

*: Significance is indicated when the median is statistically significant different from the median of the preceding stage of puberty. ($P<0.05$)

Table 7. Levels of sex steroids during sexual development in this study compared with other published studies

Study	Country	Study Type		Testosterone					Oestradiol				
				Tanner Stage 1	Tanner Stage 2	Tanner Stage 3	Tanner Stage 4	Tanner Stage 5	Tanner Stage 1	Tanner Stage 2	Tanner Stage 3	Tanner Stage 4	Tanner Stage 5
Current study	South Africa	C-S	Median IQR N	**0.05** 0.05-0.1 101	**0.2** 0.05-0.6 73	**3.9*** 1.6-8.4 38	**10.5*** 5.4-13.6 34	**12.9*** 10-18.9 7	**31.5** 23.1-45.5 99	**40.5** 29.7-50.4 74	**56.5*** 46.1-72.2 38	**79.4*** 61.5-88.5 34	**100.9*** 87.2-115.2 7
A.M. Andersson *et al.*, 1997	Danish	C-S	Median 90% PI N	**0.2** 0.2-0.9 155	**1.9*** 0.2-13.4 47	**8.4*** 0.9-21.2 27	**17.2*** 7.7-26.5 31	**21.0*** 11.3-32.3 130	**18.0** 18.0-34.0 154	**21.0*** 18.0-45.0 46	**36.0*** 18.0-85.0 25	**59.0*** 29.0-102.0 31	**71.0*** 44.0-117.0 129
T. Raivio *et al.*, 1998	Finland	L	Mean ±SD N	**0.4** 0.2-0.9 16	**1.4** 0.6-3.4 37	**3.9** 1.6-9.4 37	**18.1** 8.0-41.0 37	**35.4** 23.0-55.0					
P. M. Crofton *et al.*, 2001	Ireland	L	Median IQR N	**0.3** 0.3-0.5 90	**0.9*** 0.5-1.7 38	**6.2*** 3.0-11.2 39	**15.5** 10.2-21.0 18	**18.4** 15.8-20.0 10					
M. CHADA *et al.*, 2002	Czech Republic	C-S	Mean Min~Max N	**0.27** 0.07-0.55 11	**0.97** 0.44-1.85 8	**3.73** 1.52-7.21 12	**12.74** 8.9-18.1 10						

L, longitudinal; C-S, cross-sectional; IQR, interquartile range; N, number of boys; PI, predict interval; SD, standard deviation;

*, Significance is indicated when the median is statistically significant different from the median of the preceding stage of puberty. (P<0.05)

As mentioned before, the lower number of boys above 14 years of age group (12%) lowered statistical power for this group which might have been a limitation in the study. Another limitation in this study also noted before is that serum inhibin B which was found to be a strong marker of Sertoli cell function and spermatogenesis compared to FSH in previous studies, was not measured in this study. Additionally, the cross-sectional design only captures a single time point during pubertal development of the boys and a longitudinal design whereby each boy is followed during pubertal development would be more accurate.

CONCLUSION

The age at onset of puberty as indicated by Tanner genital stage 2 in Western Cape rural boys were late compared to boys from other settings. When late puberty approached, the delay in sexual maturation was diminished. A similar catch-up pattern was found in the height and weight increase, which were lower than CDC and WHO standards until Tanner Stage 3 and then normalized for higher Tanner Stages. The relatively low levels of testosterone in Western Cape rural boys may explain the delay in early maturational process. The high levels of FSH in Tanner Stage 4 in Western Cape rural boys may reflect a low number of Sertoli cells after pubertal proliferation. Whether such high levels of FSH in late puberty relate to reduced semen quality in adulthood requires additional study. The concerns in the delay in anthropometric and sexual maturation during the early puberty amongst boys in these communities, may also impact on reproductive ability in adulthood, initiatives to improve nutrition in Western Cape rural communities.

ACKNOWLEDGEMENT

The National Research Foundation (SA), Medical Research Council (SA) and University of Cape Town Research Committee are acknowledged for their grant support.

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APPENDIX A - CDC Growth Chart

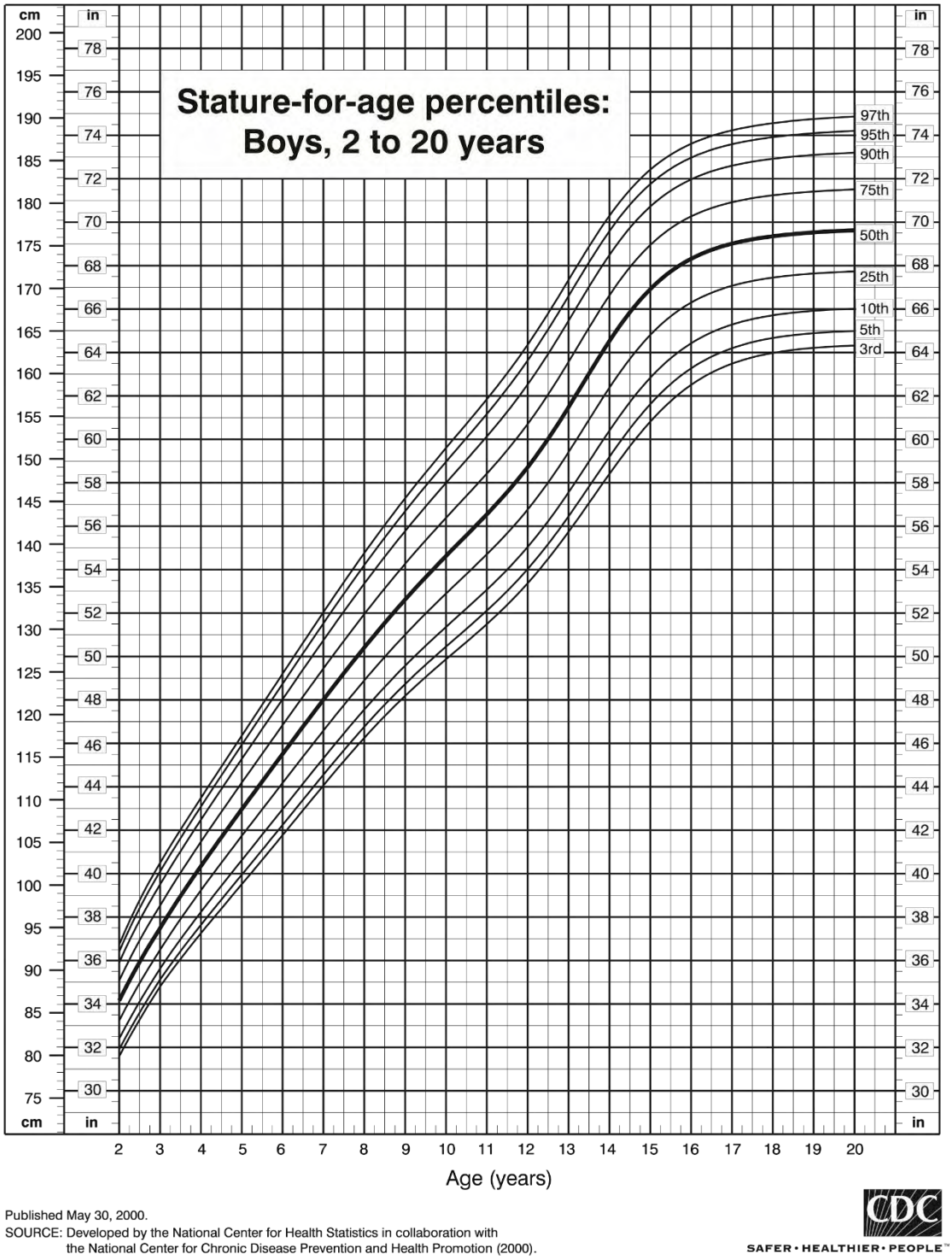

Figure 2 CDC Growth Chart - Stature

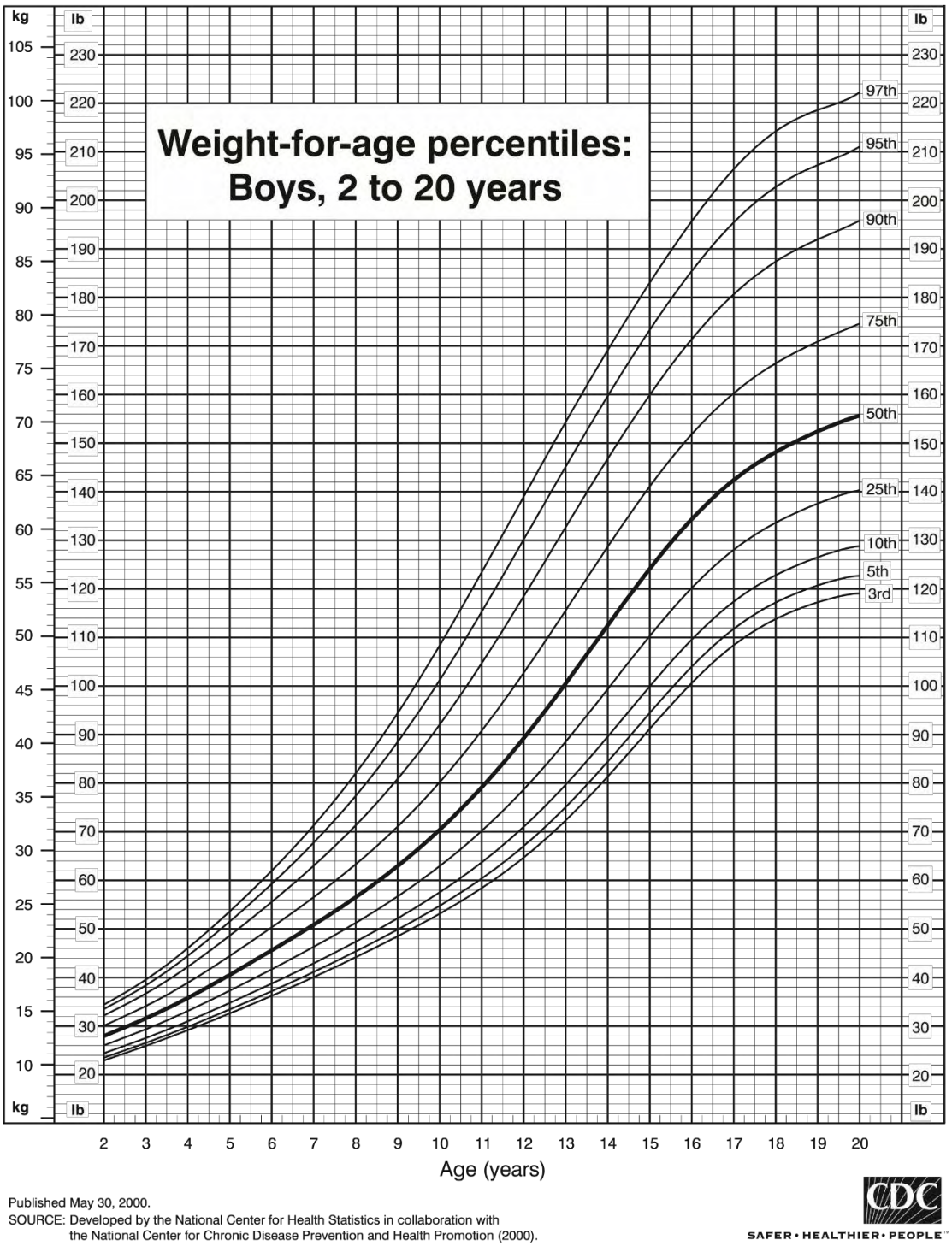

Figure 3 CDC Growth Chart - Weight

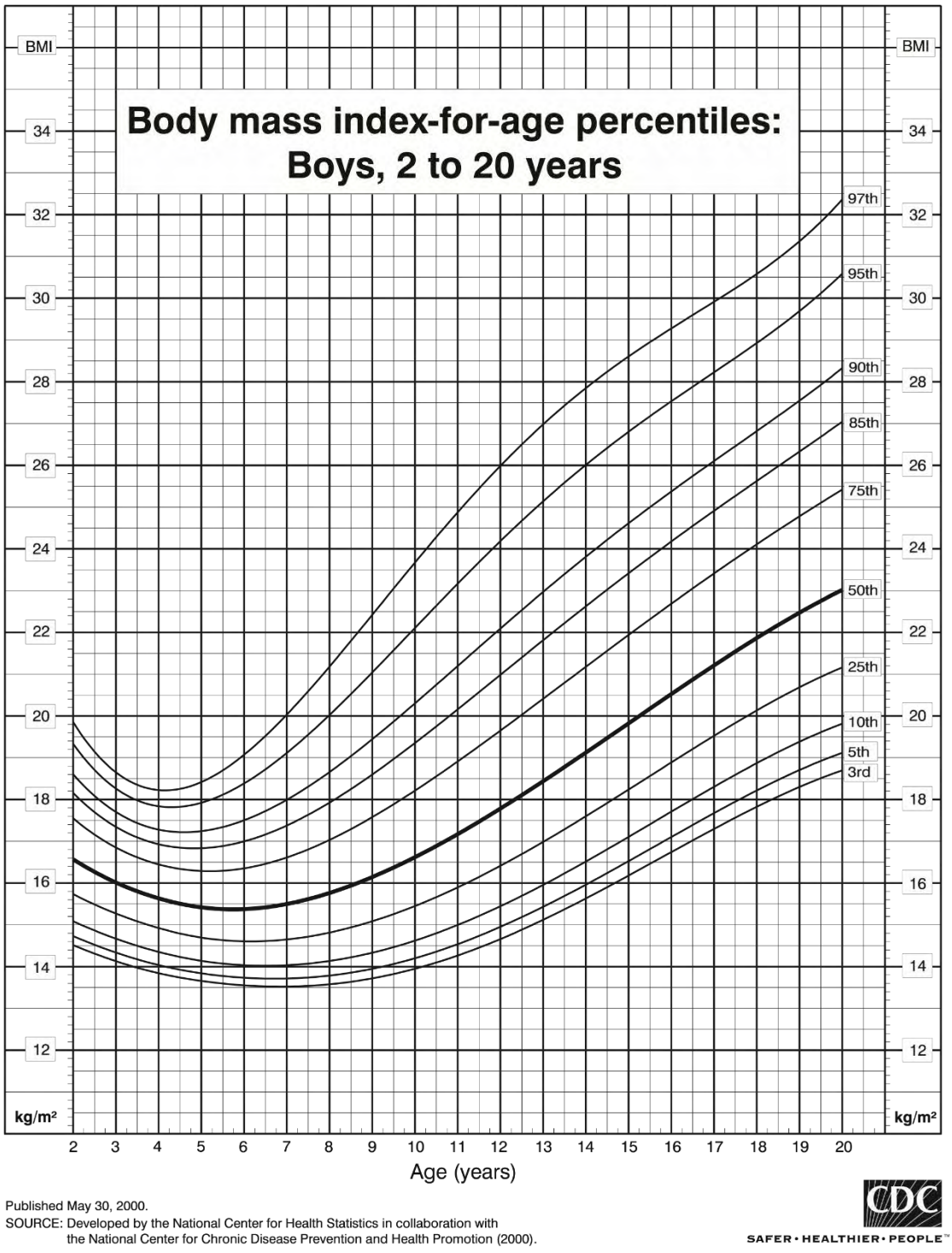

Figure 4 CDC Growth Chart - BMI

APPENDIX B - STUDY AREA

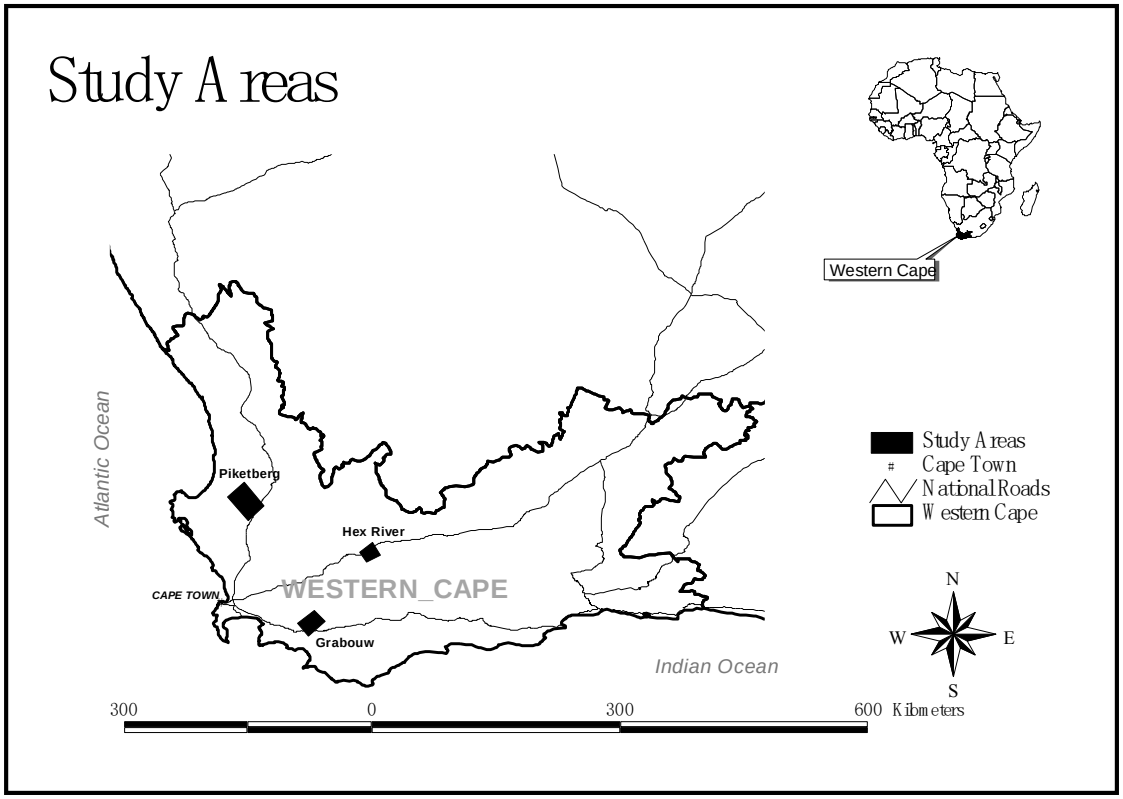

Figure 5 Location of Study Areas

APPENDIX C - TANNER STAGES ASSESSMENT DIGRAM

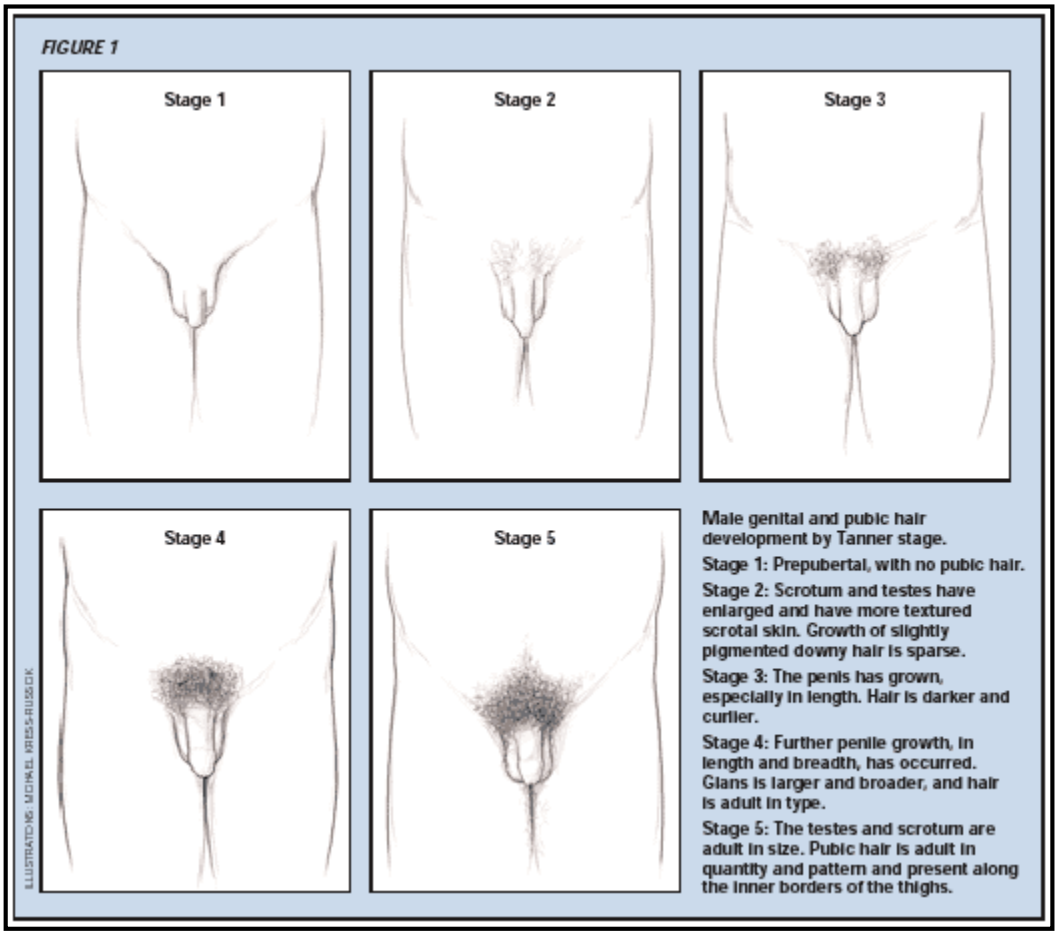

Figure 6 Tanner Stages Assessment Digram

Source: http://www.childclinic.net/pain/tanner_puberty_boys_img.gif

APPENDIX D - QUESTIONAIRE

CHILD QUESTIONNAIRE

(Male reproductive health effects due to pesticides amongst farm residents in the Western Cape)

Date _____ Room Temperature _____

Survey Number _____

Name of the Interviewer _____

Study Area _____

School _____

Source of drinking water _____

Specify the source of drinking water _____

Details of parent:

Relationship to participants: mother, father, other (circle which one is applicable)

If other, specify _____

Highest Standard/Grade passed at school: _____

Diplomas/Tertiary Education: _____ (Y/N)

Employment status _____ (yes, no, student, retired, other)

If employed, Job Title: _____

If farm worker, Exposure group: _____

(Supervisor, Sprayer/Mixer, Non- Sprayer Farmworker, Non Farmworker)

Marital Status _____

(Married, living with someone as married, widowed, divorced, separated, single with girl friend, single with no girl friend)

What is your monthly household income (in Rands)? _____

How often do your family go hungry or have no food to eat:

Never _____
Seldom____
Sometimes_____
Often_____

Details of son:

Date of birth _____ Age (_____)

Gender: ____ (Male/Female)

Birth weight:_____(kg)

Current standard/grade at school: _____

Address _____

A. GENERAL MEDICAL HISTORY

A1. How do you judge your son's health in general? _____
(Excellent, Very good, Good, Bad)

A2. Did he have/does he have:

Disease	**Yes, No, Don't Know**	**Year Diagnosed**
Diabetes		
TB		
Fits		
High Blood Pressure		
Asthma		
Heart Problems		
Back Problems		
HIV		
Foetal Alcohol Syndrome		
Other Specify:		

A3.a) Did he have /does he have any other chronic illnesses (longer than three months) apart from those listed above? __ (1 = Yes, 2 = No)

b) If yes, specify _____

A4. Has he taken any daily medication during the last 3 months? ___
(Yes, No)

A5. Has he ever been poisoned by pesticides? _____ (Yes, No, Don't know)

If yes, give details (date, name of doctor, name of hospital)

_____

B. GENITAL HEALTH HISTORY AND PUBERTY

B1. Did your son ever had mumps? ___ (Yes, No, DN)

B2. If yes, how old was he when he had mumps? _____years old

B3. Do you think your child has already entered puberty? _____ (Yes No)

If : Yes

a. At what age do you think your child entered puberty?

_____ years, _____ months

b. What was the first sign of puberty you saw in your child?

_____

If : NO (not yet entered puberty)

c. At what age do you expect your child to enter puberty?

_____ years, _____ months

d. What is the first sign of puberty you expect to see?

_____

B4. Would you say that your son's growth spurt (in height) has started yet? (A growth spurt is defined as growth in height that is faster than usual.)

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

If yes, at what age _____(years)

B5. Would you say that growth of his underarm and pubic hair has started yet?

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

If Yes, at what age? _____(years)

B6. Have you noticed any changes in his skin, especially pimples?

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

B7. Have you noticed a deepening of his voice?

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

If yes, at what age _____(years)

B8. Has he started to grow hair on his face

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

B9. Compared with other boys his age, would you say your son's physical development is:

_____

(much earlier than the other boys, somewhat earlier than the other boys, about the same as the other boys, somewhat later than the other boys, much later than the other boys)

B10. Was your son born with abnormally developed testicles? ___ (yes, no, DN)

if Yes, did he go for an operation or received medication?

_____ . What was the date he went for an operation

or received medication? _____

B11. Has your son ever had an injury, resulting in swelling/discolouring in the

testicular area? ____ (yes, no, DN)

B12. Has he ever had an operation in the testicular area?

If YES, which date?

B13. Has he been sterilized? _____(Yes, No)

B14. Has your son ever had any other diseases in the testicular area?
____ (Yes, No, Don't Know)

If "Yes", specify and give the date

_____

B15. Did your son already had his first wet dreams? _____

If yes, at what age? _____

B16. From the diagram, what stage of development do you consider your child?

Pubic hair and genital development :_____ (a, b, c, d or e)

C. LIVING HISTORY

Please answer the following questions regarding the places where your son has lived in his lifetime (C1-C16 is for current residence, Sections CA-CD is only applicable for residences before current residence starting from the most recent one)

C1 Where does he live currently? _____ (Name of town or city)

C2 For how long has he been living there? _____(years, months)

C3 Is his home located on a farm, town or city**?** _____

C3 If the place was on a farm, what kind of farm

C4 If his home is located on a farm, how far from the house is the nearest vineyard/field? _____ (meters)

C5 Are pesticides sprayed on the vineyard/field during the year? ____ (yes, no, DN)

IF No (go to C7)
IF YES, complete the following:

How many times a year are pesticides applied by means of

a) a tractor with a boom sprayer _____ (number of times a year)

b) a tractor with persons using hand or backpacks? _____ (number of times a year)

c) aeroplane _____ (number of times a year)

C6 Does the pesticides spraying come into the house? ____ (yes, no, DN)

C7 Does your son come into contact with pesticides outside the house while spraying occurs (for e.g. playing near spraying area) ? _____ (yes, no)

C8 Does your son go into in the field/vineyards soon after spraying or come into contact with sprayed surfaces? ____ (yes, no)

C9 What are the sources of drinking water at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

C10 What are the sources of water for recreational use (bathing, washing of clothes) at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

C11 Does your son play swim or play in dams/rivers? _____(yes, no)

If yes, where is the dam/river located

_____

(on farm, just outside farm, more than 100m away, out of town

C12 Does your son perform help on the farm? _____ (yes, no)

If Yes,

What does he do _____ and

How often? _____
(every day, twice a week, once a week, once a month, school holidays)

C13 Is he involved in spraying or mixing pesticides? _____ (yes, no)

C14 Does he work in the pesticide store? _____ (yes, no)

C15 Does your son come into contact with empty pesticide containers? ___(yes, no)

If yes, how _____ (for eg play, drinking water, burning)

C16 Does your son eat from the crops in the vineyard/field soon after spraying?
_____ (yes, no)

The following questions are about the place your son lived before his current home

CA1 Where did you son live before? _____ (Name of town or city)

CA2 For how long did he live there? _____(years, months)

CA3 Was that home located on a farm, town or city**?** _____
C3 If the place was on a farm, what kind of farm

CA4 If his home was located on a farm, how far from the house was the nearest

vineyard/field? _____ (meters)

CA5 Was pesticides sprayed on the vineyard/field during the year?

____ (yes, no, DN)

IF No (go to C7)
IF YES, complete the following:

CA6 Did the pesticides spraying come into the house? ____ (yes, no)

CA7 Did your son come into contact with pesticides outside the house while spraying occurs (for e.g. playing near spraying area) ? _____ (yes, no)

CA8 Did your son go into in the field/vineyards soon after spraying or come into contact with sprayed surfaces? ____ (yes, no)

CA9 What were the sources of drinking water at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA10 What were the sources of water for recreational use (bathing, washing of clothes) at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA11 Does your son play swim or play in dams/rivers? _____(yes, no)

If yes, where is the dam/river located

_____

(on farm, just outside farm, more than 100m away, out of town

C12 Did your son help on the farm? _____ (yes, no)

If Yes,

What did he do? _____ and

How often? _____
(every day, twice a week, once a week, once a month, school holidays)

CA13 Was he involved in spraying or mixing pesticides? _____ (yes, no)

CA14 Did he work in the pesticide store? _____ (yes, no)

CA15 Did your son come into contact with empty pesticide containers? ___(yes, no)

If yes, how _____ (for eg play, drinking water, burning)

CA16 Did your son eat from the crops in the vineyard/field soon after spraying?

_____ (yes, no)

The following questions are about the place your son lived before his previous home

CA1 Where did you son live before ? _____ (Name of town or city)

CA2 For how long did he live there? _____(years, months)

CA3 Was that home located on a farm, town or city**?** _____

C3 If the place was on a farm, what kind of farm

CA4 If his home was located on a farm, how far from the house was the nearest vineyard/field? _____ (meters)

CA5 Was pesticides sprayed on the vineyard/field during the year?

____ (yes, no, DN)

IF No (go to C7)
IF YES, complete the following:

CA6 Did the pesticides spraying come into the house? ____ (yes, no)

CA7 Did your son come into contact with pesticides outside the house while spraying occurs (for e.g. playing near spraying area) ? _____ (yes, no)

CA8 Did your son go into in the field/vineyards soon after spraying or come into contact with sprayed surfaces? ____ (yes, no)

CA9 What were the sources of drinking water at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA10 What were the sources of water for recreational use (bathing, washing of clothes) at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA11 Does your son play swim or play in dams/rivers? _____(yes, no)

If yes, where is the dam/river located

_____

(on farm, just outside farm, more than 100m away, out of town

C12 Did your son help on the farm? _____ (yes, no)

If Yes,

What did he do? _____ and

How often? _____
(every day, twice a week, once a week, once a month, school holidays)

CA13 Was he involved in spraying or mixing pesticides? _____ (yes, no)

CA14 Did he work in the pesticide store? _____ (yes, no)

CA15 Did your son come into contact with empty pesticide containers? ___(yes, no)

If yes, how _____ (for eg play, drinking water, burning)

CA16 Did your son eat from the crops in the vineyard/field soon after spraying?

_____ (yes, no)

The following questions are about the place your son lived before his previous home

CA1 Where did you son live before? _____ (Name of town or city)

CA2 For how long did he live there? _____(years, months)

CA3 Was that home located on a farm, town or city**?** _____

C3 If the place was on a farm, what kind of farm

CA4 If his home was located on a farm, how far from the house was the nearest vineyard/field? _____ (meters)

CA5 Was pesticides sprayed on the vineyard/field during the year?

____ (yes, no, DN)

IF No (go to C7)
IF YES, complete the following:

CA6 Did the pesticides spraying come into the house? ____ (yes, no)

CA7 Did your son come into contact with pesticides outside the house while spraying occurs (for e.g. playing near spraying area) ? _____ (yes, no)

CA8 Did your son go into in the field/vineyards soon after spraying or come into contact with sprayed surfaces? ____ (yes, no)

CA9 What were the sources of drinking water at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA10 What were the sources of water for recreational use (bathing, washing of clothes) at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA11 Does your son play swim or play in dams/rivers? _____(yes, no)

If yes, where is the dam/river located

_____

(on farm, just outside farm, more than 100m away, out of town

C12 Did your son help on the farm? _____ (yes, no)

If Yes,

What did he do? _____ and

How often? _____
(every day, twice a week, once a week, once a month, school holidays)

CA13 Was he involved in spraying or mixing pesticides? _____ (yes, no)

CA14 Did he work in the pesticide store? _____ (yes, no)

CA15 Did your son come into contact with empty pesticide containers? ___(yes, no)

If yes, how _____ (for eg play, drinking water, burning)

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_____ (yes, no)

The following questions are about the place your son lived before his previous home

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C3 If the place was on a farm, what kind of farm

CA4 If his home was located on a farm, how far from the house was the nearest vineyard/field? _____ (meters)

CA5 Was pesticides sprayed on the vineyard/field during the year?

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IF No (go to C7)
IF YES, complete the following:

CA6 Did the pesticides spraying come into the house? ____ (yes, no)

CA7 Did your son come into contact with pesticides outside the house while spraying occurs (for e.g. playing near spraying area) ? _____ (yes, no)

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CA9 What were the sources of drinking water at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam,

rain water tank, etc)

CA10 What were the sources of water for recreational use (bathing, washing of clothes) at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA11 Does your son play swim or play in dams/rivers? _____(yes, no)

If yes, where is the dam/river located

_____

(on farm, just outside farm, more than 100m away, out of town

C12 Did your son help on the farm? _____ (yes, no)

If Yes,

What did he do? _____ and

How often? _____
(every day, twice a week, once a week, once a month, school holidays)

CA13 Was he involved in spraying or mixing pesticides? _____ (yes, no)

CA14 Did he work in the pesticide store? _____ (yes, no)

CA15 Did your son come into contact with empty pesticide containers? ___(yes, no)

If yes, how _____ (for eg play, drinking water, burning)

CA16 Did your son eat from the crops in the vineyard/field soon after spraying?

_____ (yes, no)

D. HOUSEHOLD PESTICIDE EXPOSURE

D1 Do you use any pesticides in your garden or in your home (eg doom, rat poison, fleas)?

_____ (yes, no)

D2 If yes, for how long have you been using pesticides at home?

_____ (number of years)

D3 How frequently do you use pesticides at home _____
(every day, 3 times a week, once a week, once a month, less than once a month)

D4 Do you have your house fumigated?

If yes, for how long? _____(number of years)

How frequently?

_____
(every day, 3 times a week, once a week, once a month, less than once a month)

D5 Does any person in the house work with pesticides?

If yes, how many? _____

Since when has there been a person that work with pesticides? _____ (year)

Does any pesticide contaminated clothes get washed at home _____(yes,no)

If yes, does it get washed with the rest of the washing? _____ (yes, no)

D6 Does your son eat fruit or vegetables from your garden _____ (yes, no)

D7 Do you use empty pesticide containers at home for domestic purposes
If yes, what do you use them for? _____

Since when have you been using empty containers at home _____ (year)

E. DIET

E1 Does your son eat meat/fish? _____ (Yes, No)

E2 How many times a week does he eat meat/fish _____

E3 In his lifetime, how many times a week did he eat meat/fish _____

E4 Does he eat vegetables? ___ (Yes, No)

E5 How many times a week does he eat vegetables _____

E6 How many times a week does he eat soy products ____

E7 In his lifetime, how many times a week did he eat vegetables _____

E8 In his lifetime, how many times a week did he eat soy products ____

E9 Does your son like to eat nuts? __

How many times a week does he eat nuts? ____

E10 In his lifetime, how many times a week did he eat nuts? ___

E11 Was he on soya milk after birth? ___

For how long? _____

E12 Does your son eat meals provided by the school?

If yes, what do they provide? _____

Please specify the meals _____

F. MOTHERS HABITS DURING PREGNANCY

F1 When you were pregnant with this son, did you spray or mix pesticides ___?

If yes, for how many weeks____?

F2 During the pregnancy, did you work in the vineyard/orchard while pesticides were

sprayed? ____ (Yes, No)

F3 Did you work in the vineyard/orchard while pesticides were not sprayed?___
(Yes, No)

F4 During the pregnancy, did you smoke?___ (Yes, No)

If yes, how many cigarettes per day? ____

F5 During the pregnancy, did you drink alcohol?__ (Yes, No)

If yes, how many bottles per week? ____

(if papsak, estimate number of bottles)

F6 During the pregnancy, how many times a week did you eat meat/fish _____

F7 During the pregnancy, how many times a week did you eat vegetables _____

F8 During the pregnancy, how many times did you eat soya beans

or soy products ____

F9 During the pregnancy, how many times a week did you eat nuts ___

G. SMOKING AND ALCOHOL

G1 Does your son smoke currently or did he smoke before? _____ (yes, no)

If yes, for how long? _____ (number of years)

G2 Does anyone in the house smoke? _____ (yes, no)

G3 Does your son drink alcohol currently or did he drink alcohol before?

_____ (yes, no)

If yes, for how long? _____ (number of years) and how many bottles per

week? _____ (estimate if papsak)

G4 Does your take drugs or smoke dagga currently or before?

_____ (yes, no)

If yes, for how long? _____ (number of years)

APPENDIX E - CONSENT FORM

Consent to participate in a survey of investigating health effects due to occupational and environmental pesticide exposures on male farm residents in the rural Western Cape

1. **Title of research project**

Male reproductive effects due to pesticide exposure in the Western Cape, South Africa

2. Names of the researchers

Mohamed Aqiel Dalvie (BSc, Honours, MSc, PhD)
Algernon Africa (BTech)
Vicky Major (
Leslie London (MBChB, Honours, MD)
Eugene Cairncross (BSc, Honours, PhD)

3. Purpose of research

The University of Cape Town is conducting this survey to investigate the reproductive health effects of pesticides on young boys and men in the Western Cape. This will be of benefit to men
and boys living in farming areas and who are exposed to pesticides either at work or in the environment.

4. Description of the research project

We will conduct tests on one day. Your son will be required to produce a urine and blood sample
and undergo a physical examination and you will complete a questionnaire.

a) **Questionnaire:**A member of our study team will interview you in privacy to complete the questionnaire. You will be asked questions about general personal information about your son, his general medical health, genital health history and lifetime environmental exposure to pesticides.

b) **Urine sample:** Your son has to produce a urine sample (in privacy) in a plastic container and give it to the nurse. The sample will be analysed for pesticides.

c) **Blood sample:** A nurse will draw 10 ml blood from a vein on your son's arm. The blood will be analysed for pesticides and for the levels of hormones.

d) **Physical examination:** A doctor will assess your son's reproductive health.

5. Risks and discomforts of the research

a) **From the blood tests**. A single needle stick will be felt when the blood is taken. Sometimes a small bruise may occur from the needle stick, but this is minor and will heal quickly. The total amount of blood taken is quite small and the body will quickly replace it. Blood samples will be used only to measure pesticides and reproductive hormones and will be destroyed at the end of the study.

b) **From the questionnaire.**
There are minimal risks associated with completing the questionnaire. The only risk is loss of confidentiality about personal information but the data will be seen only by study personnel. All reports will present aggregate data in which individuals will not be identifiable.

6. Expected benefits to you and others

A doctor will examine your son's reproductive health.

Refreshments will be provided as compensation for time in participating in the study.

This study on the reproductive health effects of pesticides will benefit men and boys living in farming areas and who are exposed to pesticides either at work or in the environment. Steps can be taken to reduce or prevent exposure to the pesticides or the pesticide can be banned. The blood and urine results can be used to develop ways in which the amount of pesticides in your body can be monitored.

7. Costs to you resulting form participation in the study

The study is offered at no cost to you.

8. Confidentiality of information collected

Study participants will not be personally identified in any reports on this study. The records will be kept confidential to the extent provided by law. The records, including any identification information, will be destroyed after the results have been fully analysed.

9. Documentation of the consent

One copy of this document will be kept together with our research records on this study. A second copy will be given to you to keep.

10. Contact person.

You may contact the following person for answers to further questions about the research, your rights, or any injury you may feel is related to the study.

Name of person: MA Dalvie (The principal investigator) - telephone 021 4066610
Name of person: Lamees Emjedi (Ethics administrator) - telephone 021 4066492

11. Voluntary nature of participation

Your son's participation in this project is voluntary. Subsequent to your consent, you may refuse your son to participate in or withdraw from the study at any time without penalty or loss of benefits to which you may otherwise be entitled.

12. Consent of the participant

I have read the information given above. I understand the meaning of this information. I hereby consent for my son to participate in the study.

_____

_____

Printed name of parent/ participant (adolescent or adult)
signature

_____

_____

Date

_____ _____

Interviewers (print) signature Date

_____ _____

Witness (print) signature Date

Date:_____

Study Number _____

APPENDIX F - INSTRUCTIONS FOR AUTHORS

Research article

Criteria

Research articles should report on original primary research, but may report on systematic reviews of published research provided they adhere to the appropriate reporting guidelines which are detailed in our editorial policies. Please note that non-commissioned pooled analyses of selected published research will not be considered.

Preparing your manuscript

Title page

The title page should:

- present a title that includes, if appropriate, the study design e.g.:
  - "A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"
  - or for non-clinical or non-research studies a description of what the article reports
- list the full names, institutional addresses and email addresses for all authors
  - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the "Acknowledgements" section in accordance with the instructions below
- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

- **Background:** the context and purpose of the study
- **Methods:** how the study was performed and statistical tests used
- **Results:** the main findings
- **Conclusions:** brief summary and potential implications
- **Trial registration:** If your article is a systematic review or reports the results of a health care intervention on human participants, it must be registered in an appropriate registry

and the registration number and date of registration should be in stated in this section. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

Declarations

List of abbreviations
If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Ethics approval and consent to participate
Manuscripts reporting studies involving human participants, human data or human tissue must:

- include a statement on ethics approval and consent (even where the need for approval was waived)
- include the name of the ethics committee that approved the study and the committee's reference number if appropriate

Studies involving animals must include a statement on ethics approval.

See our editorial policies for more information.

If your manuscript does not report on or involve the use of any animal or human data or tissue, this section is not applicable to your submission. Please state "Not applicable" in this section.

Consent for publication
If your manuscript contains any individual person's data in any form, consent to publish must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent to publish. You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication).

If your manuscript does not contain any individual persons data, please state "Not applicable" in this section.

Availability of data and materials
For all journals, BioMed Central strongly encourages all datasets on which the conclusions of the manuscript rely to be either deposited in publicly available repositories (where available and appropriate) or presented in the main paper or additional supporting files, in machine-readable format (such as spreadsheets rather than PDFs) whenever possible. Please see the list of recommended repositories in our editorial policies.

For some journals, deposition of the data on which the conclusions of the manuscript rely is an absolute requirement. Please check the Criteria section for this article type (located at the top of this page) for journal specific policies.

For all journals, authors must include an "Availability of data and materials" section in their article detailing where the data supporting their findings can be found. If you do not wish to share your data, please state that data will not be shared, and state the reason.

For information on how to cite your data and format this section see preparing your manuscript.

Competing interests

All financial and non-financial competing interests must be declared in this section. See our editorial policies for a full explanation of competing interests. If you are unsure whether you or any of your co-authors have a competing interest please contact the editorial office.

Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. Guidance and criteria for authorship can be found in our editorial policies.

Acknowledgements

Please acknowledge anyone who contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials.

Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements section.

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Endnotes

Endnotes should be designated within the text using a superscript lowercase letter and all notes (along with their corresponding letter) should be included in the Endnotes section. Please format this section in a paragraph rather than a list.

References

For guidance on the appropriate use of references, see our editorial policies.

For information on how to format your references, see preparing your manuscript.

Illustrations and figures (if any)

For information on how to format your figures, see preparing your manuscript.

Tables and captions

For information on how to format your tables, see preparing your manuscript.